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Comparison of the Sensititre YeastOne colorimetric antifungal panel and Etest with the NCCLS M38-A method to determine the activity of amphotericin B and itraconazole against clinical isolates of *Aspergillus* spp.

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***Objective*: The evaluation of the Sensititre YeastOne and Etest methods for determining susceptibility to amphotericin B and itraconazole by comparing the MICs obtained by these methods at different times of reading with those of the M38-A broth microdilution method.**

***Methods*: Sixty-three clinical isolates of *Aspergillus* spp. (23 *Aspergillus flavus*, 24 *Aspergillus fumigatus*, nine *Aspergillus niger*, three *Aspergillus glaucus*, two *Aspergillus terreus* and two *Aspergillus flavipes*) were assayed. Two itraconazole-resistant strains (NCPF7100 and 7099) were also included.**

***Results*: Itraconazole MICs for the two resistant strains were >4 mg/L by the three methods. The overall agreement (±2 $\log_2$) between M38-A (48 h) and colorimetric (48 h) method was 93.4% for amphotericin B and 90.2% for itraconazole. By the Etest, the best agreement with M38-A was obtained when readings were made at 24 h: 88.5% for amphotericin B and 67.2% for itraconazole. Etest MICs were higher for all species except *A. niger*.**

***Conclusions*: The colorimetric method appears to be a suitable alternative procedure for antifungal susceptibility testing of *Aspergillus* spp. and is able to detect resistance to itraconazole. The range of MICs for amphotericin B by Etest is wider and for some strains is >16 mg/L, suggesting that this method could be useful for detecting resistant strains as occur in yeasts.**

Keywords: *Aspergillus* spp., susceptibility testing, MIC, Sensititre YeastOne

Introduction

In recent years, opportunistic life-threatening fungal infections have increased and a variety of moulds have emerged as important pathogens in immunocompromised hosts. Among these pathogenic moulds, *Aspergillus* species are frequently responsible for these infections; *Aspergillus fumigatus* is the most common species causing pulmonary disease. The majority of *Aspergillus* isolates are susceptible to both amphotericin B and itraconazole, the primary drugs available for the systemic treatment of these infections. Resistance to these drugs has been reported both *in vitro* and *in vivo* in *A. fumigatus*[1–4] and resistance to amphotericin B among isolates of *Aspergillus terreus*,[5] as well as treatment failure in invasive diseases caused by this species.[6] This situation warrants susceptibility testing. In 2002, the NCCLS published approved guidelines (M38-A)[7] for susceptibility testing of filamentous moulds; however, the method is not a practical testing tool for a clinical laboratory, as it is very time consuming. Sensititre YeastOne and the Etest are commercially available methods, which have been assayed for antifungal susceptibility testing of yeasts, the former with good correlation with the reference method (M27-A) (>90%) and the latter being variable depending on the yeast/antifungal combination.[8–10] Very few data have been published regarding the application of Sensititre YeastOne to filamentous fungi.[11,12] In addition, the results of agreement between Etest and M38-A methods are controversial, in part due to

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different MIC endpoints and incubation times being applied for itraconazole: apparent reduction of growth and 48 h incubation,[4,13] apparent reduction of growth and 24 h incubation[14] or 100% growth inhibition and 24 h incubation.[15] In contrast, the agreements of the colorimetric method with the M38-A are >90%.[11,12]

The present study evaluated the Sensititre YeastOne and Etest methods for determination of susceptibility to amphotericin B and itraconazole. MICs obtained by these methods at different times of reading were compared with those obtained by the NCCLS M38-A broth microdilution method.

Materials and methods

Antifungal agents

Itraconazole in powder form was provided by Janssen Pharmaceutica (Beerse, Belgium) and amphotericin B from Sigma–Aldrich Quimica SA (Madrid, Spain). The drugs were dissolved in dimethyl sulphoxide (DMSO; Sigma–Aldrich Quimica SA) at a concentration of 1600 mg/L. Additive two-fold dilutions of the drugs were made in DMSO and diluted 1:100 in the assay medium. The final concentration of DMSO in the medium was 1%.

Organisms

A total of 63 *Aspergillus* clinical isolates, comprising 23 isolates of *Aspergillus flavus*, 24 *A. fumigatus*, nine *Aspergillus niger*, three *Aspergillus glaucus*, and two each of *Aspergillus flavipes* and *A. terreus* were used in this study. These isolates were recovered from clinical specimens (ear, sputum, wound, bronchial alveolar lavage and bronchial aspirate) of 63 adult patients between 1995 and 1998. Repeated isolates from the same patient were excluded. Culture, identification and preservation of strains were carried out by standard methods.[16,17] *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains for susceptibility testing procedures and *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were used as reference strains. Two itraconazole-resistant strains, *A. fumigatus* NCPF 7100 and NCPF 7099, were included to check the ability of the methods to detect resistance to itraconazole.[1,2]

Susceptibility testing

Broth microdilution was performed according to the NCCLS document M38-A.[7] Inoculum suspensions were prepared from 7 day cultures grown on potato dextrose agar (Oxoid) at 35°C and adjusted spectrophotometrically (530 nm) to optical densities that ranged from 0.09 to 0.11. Plates were inoculated with 100 µL of these suspensions diluted to 1:50 in RPMI 1640. The final inoculum varied between 0.5×10^4 and 5×10^4 cfu/mL, as demonstrated by quantitative colony counts. The final volume was 200 µL. Concentrations tested ranged from 16 to 0.03 mg/L. Drug- and cell-free controls were included. Readings were made after 48 and 72 h of incubation at 35°C by visually comparing the turbidity of each well with that of the control well (without drug), with the aid of a reading mirror. The MIC endpoint for itraconazole was the lowest drug concentration that showed prominent growth inhibition. For amphotericin B the MIC was defined as the lowest concentration that showed complete inhibition of growth.

Sensititre YeastOne panels (Trek Diagnostic Systems Ltd, East Grinstead, UK) containing serial two-fold dilutions of the drugs (16–0.016 mg/L) were stored at room temperature until testing was performed. Inoculum suspensions were prepared in the same way as for the M38-A method and the adjusted suspensions were diluted 1:100 in YeastOne RPMI (American BioOrganics, Buffalo, NY, USA). The dried YeastOne panels were rehydrated, and 100 µL of the working suspension was dispensed into each well with a multichannel pipetting device. The panels were covered with seal strips and incubated at 35°C for 72 h, and were read after 48 and 72 h of incubation. Colorimetric MICs were interpreted as the lowest concentration of antifungal solutions changing from red (growth) to blue (no growth) for amphotericin B, or changing from red to purple (growth inhibition) or blue for itraconazole.

Etest strips (AB Biodisk, Solna, Sweden) and RPMI agar plates with 2% of glucose (RPMIG) (Remel, Lenexa, KS, USA) were purchased from IZASA (Barcelona, Spain). Strips were stored at –20°C until use. Etest was performed in accordance with the manufacturer's instructions. Spore suspensions were prepared in the same way as for the M38-A and adjusted to 0.5 McFarland turbidity units. A swab was dipped into the cell suspensions and streaked onto the entire surface of the RPMIG plate in three directions. The plates were allowed to dry for at least 15 min before the strips were placed on the medium surface. After incubation of the plates at 35°C for 24 and 48 h, MICs were read. Amphotericin B MIC was read as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip. The itraconazole MIC was the lowest concentration in which there was 80% inhibition of growth, as described and illustrated in the Etest technical guide for antifungal susceptibility testing (AST No. 10).

Data analysis

MIC ranges and the corresponding geometric mean values (GM MIC) were obtained for each species, incubation time and drug combination, by each method tested. The MIC_{90}s were determined for species for which five or more isolates were available. Because the Etest scale has a continuous gradient of concentrations instead of the two-fold dilutions scheme of the broth microdilution methods, the MICs determined by the Etest were raised to the next two-fold concentration that matched the microdilution scheme for the sake of comparison, but not the GM MICs. For those strains in which the difference in MICs with the M38-A was more then two two-fold dilutions, MICs were repeated by the three methods. The comparisons analysed were: (i) 48 h MIC pairs by colorimetric method versus 48 h M38-A MIC; (ii) 72 h colorimetric MIC versus 48 h M38-A MIC; (iii) 24 h Etest-MIC versus 48 h M38-A MIC; and (iv) 48 h Etest MIC versus 48 h M38-A MIC. Discrepancies among MIC endpoints of no more than two two-fold dilutions were used to calculate the percentage of agreement, and this was determined for each combination of isolate, drug and incubation time.

Results

The MICs for quality control strains and reference strains were within the published range at both 24 and 48 h[7,13,18] for all three methods. MICs for the reference itraconazole-resistant *A. fumigatus* were ≥4 mg/L for itraconazole by the three methods after 48 h of incubation and 24 h for Etest. The modal MIC of amphotericin B for the NCPF 7100 strain was 0.5, 1 and 4 mg/L by M38-A, Sensititre YeastOne and Etest, respectively, and that of NCPF 7099 was 0.25, 1 and 1.5 mg/L, respectively, after 48 h of incubation. None of these strains showed enough growth to be able to be read in Sensititre YeastOne after 24 h of incubation. Amphotericin B MIC by the Etest for NCPF 7099 was 0.75 mg/L at 24 h and 1.5 mg/L for NCPF 7100 strain.

Effect of incubation time on MICs

All the strains showed good growth after 48 h of incubation, except one strain of *A. niger* that did not grow in RPMI 1640 broth medium and another strain of the same species that required 72 h to grow in the same medium; these strains grew in this medium with glucose. The

Table 1. Amphotericin B MIC ranges for 63 strains of *Aspergillus* spp. as determined by M38-A, YeastOne and Etest

		MIC (mg/L)								
		M38-A			YeastOne			Etest		
Organism (no. tested)	Incubation time (h)	range	MIC_{90}	GM MIC	range	MIC_{90}	GM MIC	range	MIC_{90}	GM MIC
A. flavus (23)	24	ND	ND	ND	ND	ND	ND	0.12–4	1	0.42
	48	0.06–2	1	0.29	0.25–1	1	0.55	0.12–16	4	1.29
	72	0.12–2	1	0.41	0.5–2	1	0.87	ND	ND	ND
A. fumigatus (24)	24	ND	ND	ND	ND	ND	ND	0.008–4	2	0.27
	48	0.06–2	1	0.36	0.25–1	1	0.5	0.25–16	8	1.1
	72	0.25–2	1	0.58	0.5–1	1	0.77	ND	ND	ND
A. niger[a] (9)	24	ND	ND	ND	ND	ND	ND	0.06–0.25	0.25	0.13
	48	0.03–0.5	0.5	0.16	0.25–0.5	0.5	0.29	0.12–0.5	0.5	0.19
	72	0.06–1	1	0.25	0.25–1	1	0.57	ND	ND	ND
Other *Aspergillus* spp.[b] (7)	24	ND	ND	ND	ND	ND	ND	0.12–4	4	0.71
	48	0.06–1	1	0.34	0.25–2	2	0.67	0.12–16	16	1.42
	72	0.06–1	1	0.45	0.5–4	4	1.21	ND	ND	ND
All isolates (63)	24	ND	ND	ND	ND	ND	ND	0.008–4	1	0.32
	48	0.06–2	1	0.3	0.25–2	1	0.49	0.12–16	8	1.04
	72	0.06–2	1	0.46	0.25–4	1	0.79	ND	ND	ND

ND, not determined.
[a]One strain did not grow in RPMI broth and another grew at 72 h.
[b]Three *A. glaucus*, two *A. flavipes* and two *A. terreus*.

MICs of amphotericin B by the M38-A and colorimetric methods increased one dilution for ~50% of the strains when readings were made at 72 h compared with those obtained at 48 h. MICs for only two strains increased two dilutions by both methods, and one increased three dilutions by the M38-A method. For itraconazole, by the M38-A method, the MICs for 93.4% of the strains were the same at 48 and 72 h, and the other 6.6% increased one dilution step. By the colorimetric method the MICs of itraconazole for 57.1% of the strains were the same at 48 and 72 h. For the other strains MICs increased one dilution at 72 h, except for in two instances of a two dilution increase. By Etest, for 36.5% of the strains the itraconazole MIC did not change with time of incubation, 49.2% of the strains had an increase two or less dilutions, and for 14.3% the increase was three or more dilutions. Interestingly, although the increase was more than three dilutions, MICs were still below 1 mg/L. Etest MICs of amphotericin B increased more than two dilutions for 18.0% of the strains from 24 to 48 h.

MIC results

The MIC ranges, MIC_{90} and GM MIC values for the 63 isolates of *Aspergillus* spp. determined by the M38-A, colorimetric and Etest methods for the two drugs and the time of reading, are summarized in Tables 1 and 2.

By the M38-A method, all the strains tested were inhibited with ≤2 mg/L of amphotericin B and ≤0.25 mg/L of itraconazole, independent of time of reading.

By the colorimetric method, MICs of itraconazole were ≤0.5 mg/L and those of amphotericin B ≤2 mg/L, except for two strains of *A. flavipes*, which were 2 mg/L at 48 h and increased to 4 mg/L at 72 h of incubation.

In general, MIC ranges by Etest were broader and MICs were always higher for both drugs, except for *A. niger*. For one strain each of *A. flavus* and *A. fumigatus*, and two strains of *A. flavipes*, MICs of amphotericin B by Etest at 24 h were 4 mg/L and shifted to 16 mg/L at 48 h. For these strains, the MICs by the M38-A and colorimetric methods were 1 mg/L for *A. flavus* and *A. fumigatus*, independent of the time of reading; but for *A. flavipes*, the MIC by colorimetric method was 4 mg/L at 72 h. In contrast, *A. niger* was the only species that yielded lower amphotericin B MICs by Etest (GM MIC 0.13 versus 0.16 mg/L). The Etest MICs of itraconazole for 87.5% of the strains were ≤0.5 mg/L. The strains that required ≥1 mg/L of itraconazole to be inhibited belonged to the species *A. fumigatus* (five strains), *A. flavus* and *A. niger* (one strain each). These strains were inhibited by ≤0.12 mg/L by the M38-A and colorimetric methods.

Agreement of the Sensititre YeastOne and Etest with M38-A method

The agreement between methods for amphotericin B and itraconazole was dependent on both the time of reading and species being evaluated (Table 3), and was better when comparing colorimetric and M38-A methods than when comparing Etest and M38-A, for both amphotericin B and itraconazole. The overall agreement between colorimetric (48 h) and M38-A methods (48 h) for amphotericin B was 93.4% and for itraconazole 90.2%. The overall agreement between Etest (24 h) and M38-A (48 h) method was 88.5% for amphotericin B. The highest and lowest agreements obtained for amphotericin B by species were for *A. niger* (100%) and *A. flavus* (60.9%). For itraconazole the overall agreement between Etest and M38-A was 67.2%. Five strains of *A. fumigatus* and one each of *A. flavus* and *A. niger*, for which the MICs were ≥1 mg/L at 24 h by the

Table 2. Itraconazole MIC ranges for 63 strains of *Aspergillus* spp. as determined by M38-A, YeastOne and Etest

		MIC (mg/L)								
		M38-A			YeastOne			Etest		
Organism (no. tested)	Incubation time (h)	range	MIC_{90}	GM MIC	range	MIC_{90}	GM MIC	range	MIC_{90}	GM MIC
A. flavus (23)	24	ND	ND	ND	ND	ND	ND	0.03–2	0.25	0.13
	48	0.016–0.25	0.06	0.03	0.06–0.25	0.12	0.08	0.12–2	0.5	0.31
	72	0.016–0.25	0.12	0.03	0.06–0.25	0.25	0.12	ND	ND	ND
A. fumigatus (24)	24	ND	ND	ND	ND	ND	ND	0.06–4	1	0.34
	48	0.016–0.25	0.12	0.06	0.06–0.25	0.12	0.1	0.12–6	2	0.48
	72	0.016–0.25	0.12	0.06	0.06–0.25	0.25	0.12	ND	ND	ND
A. niger[a] (9)	24	ND	ND	ND	ND	ND	ND	0.016–0.5	0.5	0.07
	48	0.016–0.25	0.25	0.07	0.06–0.25	0.25	0.1	0.12–2	2	0.51
	72	0.016–0.25	0.25	0.07	0.06–0.5	0.25	0.15	ND	ND	ND
Other *Aspergillus* spp.[b] (7)	24	ND	ND	ND	ND	ND	ND	0.03–0.5	0.5	0.14
	48	0.016–0.12	0.12	0.03	0.016–0.25	0.25	0.05	0.03–2	2	0.25
	72	0.016–0.12	0.12	0.03	0.03–0.25	0.25	0.09	ND	ND	ND
All isolates (63)	24	ND	ND	ND	ND	ND	ND	0.016–4	1	0.16
	48	0.016–0.25	0.12	0.04	0.016–0.25	0.12	0.09	0.016–6	2	0.36
	72	0.016–0.25	0.12	0.05	0.016–0.5	0.25	0.12	ND	ND	ND

ND, not determined.
[a]One strain did not grow in RPMI broth and another grew at 72 h.
[b]Three *A. glaucus*, two *A. flavipes* and two *A. terreus*.

Etest, yielded MICs ≤0.12 by the colorimetric and M38-A methods at 48 h. Agreements were always greater for amphotericin B, whatever method and time of reading compared. By Etest, the best agreement values with the M38-A were obtained when readings were made at 24 h.

Discussion

The results of the present study with respect to susceptibility of *Aspergillus* spp. to antifungal agents show differences depending on the method used and the species tested. For instance, by the M38-A method 96.8% of the strains were inhibited by <1 mg/L of amphotericin B and 100% of the strains by ≤0.25 mg/L of itraconazole. These results are comparable to those reported previously.[19,20] By the colorimetric method, all strains were inhibited by 2 mg/L of amphotericin B if readings were made at 48 h, or by 4 mg/L if made at 72 h. For itraconazole, practically no differences were observed among broth microdilution methods, all strains being inhibited by 0.5 mg/L. The MICs by the Etest were higher for both drugs in all species tested, with the exception of *A. niger*, for which the MICs of amphotericin B were lower in five of the nine strains assayed.

The agreement between results obtained by the colorimetric method and Etest with the M38-A depends on the time of reading compared and the species. Agreements for the two agents were higher for the colorimetric method (>90%) than for the Etest (88.5% for amphotericin B and 67.2% for itraconazole). The reason for the low agreement obtained for itraconazole could be the MIC endpoint used. It has been reported, in a collaborative study to determine the optimal susceptibility test conditions for detecting resistance to azoles in *Aspergillus* spp., that agreement between MICs for itraconazole improves when applying MIC endpoints of 100% of inhibition of growth.[21] This low agreement could also be due to the low reproducibility of the Etest, 73.2% and 80.4% at 24 and 48 h, respectively, for itraconazole, as reported by Meletiadis *et al.*,[12] although we have not formally studied the reproducibility of this. On the other hand, low percentages of correlation for itraconazole have also been reported when performing MICs by the Etest in yeast.[10]

MICs (48 h) by the colorimetric method were comparable to those obtained by the M38-A for both amphotericin B and itraconazole. The percentage agreement between the results of these methods for amphotericin B was 100% for *A. niger* and >85% for the other species tested. The best agreement for itraconazole between M38-A (48 h) and colorimetric (48 h) was for *A. niger* and *Aspergillus* spp. at 100% each. For the other species, agreement depended on time compared, although the best agreement was obtained when comparing MICs at 48 h. Meletiadis *et al*.[12] suggested that prolonging the incubation time in the colorimetric method to 72 h could improve the agreement. The present study demonstrates that this is not the case. Our results are similar to those reported by others,[11,12] although those investigators assayed only 17 isolates of *A. fumigatus*[11] and 25 strains, five from each of five species of *Aspergillus.*[12]

Etest and M38-A have previously been evaluated by other authors;[4,13–15] however, our results are more in accordance with those reported by Espinel-Ingroff[15] and Meletiadis *et al.*[12] In agreement with these authors, we found lower agreements for both drugs when MICs were determined by Etest at 48 h. By species, *A. niger* had the best agreement for both antifungal agents (100%), at either 24 or 48 h; the same percentage agreement for this species has been reported by others.[13–15] In contrast, we obtained lower agreements for *A. fumigatus* for both amphotericin B and itraconazole. Like Meletiadis *et al*.,[12] we also found increases in MIC values up to six step dilutions for some strains. Perhaps if 100% inhibition of growth were applied as the

Table 3. Agreement between the M38-A (48 h) method and the colorimetric or Etest method for amphotericin B and itraconazole after 24, 48 and 72 h of incubation

Organism (no. tested)	Incubation time (h)	% agreement for colorimetric method (± 2 $\log_2$ dilutions)		% agreement for Etest method (± 2 $\log_2$ dilutions)	
		amphotericin B	itraconazole	amphotericin B	itraconazole
A. flavus (23)	24	ND	ND	95.65	60.9
	48	91.3	78.3	60.9	26.1
	72	68	56.52	ND	ND
A. fumigatus (24)	24	ND	ND	79.2	66.6
	48	95.8	95.8	79.2	45.8
	72	87.5	87.5	ND	ND
A. niger (7)	24	ND	ND	100	100
	48	100	100	100	71.4
	72	85.7	85.7	ND	ND
Aspergillus spp[a] (7)	24	ND	ND	85.7	57.2
	48	85.7	100	71.4	57.2
	72	71.4	5.7	ND	ND
Overall (61)	24	ND	ND	88.52	67.2
	48	93.4	90.2	73.8	42.62
	72	80.3	75.4	ND	ND

ND, not determined.
[a]Three *A. glaucus*, two *A. flavipes* and two *A. terreus*.

endpoint for itraconazole by the M38-A method, better agreement could be obtained, as mentioned above.

As a part of this evaluation, two known itraconazole-resistant strains[1–3] were included. Both strains were resistant by the three methods assayed (MIC >16 mg/L), suggesting that colorimetric, Etest and M38-A methods are able to detect resistance to itraconazole *in vitro*.

In conclusion, on the basis of data from this study the colorimetric method has potential value for the performance of susceptibility tests in filamentous fungi. The greatest disadvantage of the Sensititre YeastOne was that only amphotericin B and itraconazole could be used. However, the incorporation of voriconazole, the new triazole recently licensed to treat serious invasive aspergillosis and candidosis, makes it more suitable for use. With respect to the Etest, the fact that the range of MICs obtained for amphotericin B was wider, and for some strains >16 mg/L, suggests that it could be useful for the detection of resistant strains such as occur in yeasts.[22,23] However, more studies are required to determine which methods show the best agreement with *in vivo* results.

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