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In Vitro Susceptibility Testing of *Aspergillus* and Non-*Aspergillus* Filamentous Moulds to Antifungal Agents: Evaluation of Three Different Methods

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Key words: Aspergillus; filamentous fungi; antifungals; resistance; susceptibility testing.

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Abstract

Aim: The aim of this study was to evaluate the suitability of Sensititre YeastOne (SYO) method for susceptibility profiles' determination of non-*Aspergillus* moulds and of E-test for *Aspergillus* spp. and non-*Aspergillus* moulds, in comparison with the M38-A reference broth microdilution (BMD) method.

Material and Methods: A total of 33 clinical isolates of filamentous fungi were tested.

Results: The agreement between E-test and BMD at ± 2 dilutions was 82.4%, 83.3% and 82.4% for amphotericin B, itraconazole and voriconazole, respectively. The agreement between SYO and BMD at ± 2 dilutions was 76.5%, 66.7% and 88.2% for amphotericin B, itraconazole and voriconazole, respectively. The majority of differing results are due to higher MICs with the reference method. In particular, SYO was unable to detect some of the potentially amphotericin B resistant strains. We found that both E-test and SYO method were reproducible and served as suitable methods for antifungal susceptibility testing of moulds.

Conclusion: In conclusion, both E-test and SYO method are promising, but require further investigation to identify the optimum conditions for their use in testing of susceptibility profiles of filamentous fungi to antifungal agents.

Introduction

The impact and severity of fungal infections have grown in recent decades and now involve a wide range of fungal pathogens as etiological agents. During this period, the number of immunocompromised patients has markedly increased [1]. Many predisposing factors have contributed to this increase such as use of new and more aggressive therapies for treatment of solid tumours, haematological malignancies, long-term therapy with corticosteroids and broad-spectrum antibiotics, the increasing number of patients who undergo organ transplantation, and finally the spread of AIDS [2]. The management of fungal infections is affected by the ability to carry out rapid and effective etiological diagnosis and availability of antifungal agents with proper spectrum of activity [3]. Delayed diagnosis and antifungal treatment contribute significantly to the high mortality rates

associated with invasive fungal infections [4], whereas early intervention with antifungal drugs may result in more effective management of high-risk patients [5].

Knowledge of potential causative organisms is required to aid the diagnostic process, mainly in situations where systemic fungal infection is suspected but the clinical presentation is nonspecific [6]. The exact identification of the etiological agent has become essential in light of the increased use of prophylactic regimens that predispose the patients' development of fungal infection and selection of resistant fungal species, such as *Candida glabrata*, *C. krusei*, *Aspergillus terreus*, *Scedosporium* species and *Zygomycetes*, many of which are intrinsically resistant to the available antifungal agents [7].

It is therefore very important to perform in vitro antifungal susceptibility testing (AFST) which should provide useful information for appropriate selection of the most active antifungal therapy against different etiological agents, as well as to predict treatment outcome or explain some resistance cases [8]. In 2002, the CLSI (Clinical and Laboratory Standards Institute, formerly NCCLS) published approved guidelines (M38-A) for broth microdilution susceptibility testing of *Aspergillus* spp. and other rapidly growing filamentous moulds (BMD) [9]. With the establishment of this reference method, there is now an opportunity to validate alternative methods for testing moulds against various systemically active antifungal agents. However this method is not a practical testing tool for a busy microbiology laboratory, because it is time-consuming and cumbersome [10].

Therefore, there is a great need for an easier and reproducible method for in vitro AFST of filamentous fungi. Alternatives to the CLSI method are E-test and Sensititre YeastOne (SYO) antifungal panel, two commercial methods that have been evaluated for yeasts and moulds [11-16]. SYO is a commercial colorimetric panel, that consists of a disposable tray which contains dried serial dilutions of seven antifungal agents in individual wells, which also contain an oxidation-reduction indicator (AlamarBlue) to generate clear-cut endpoints based on a visually detectable change in colour from blue to pink, and this indicates the antifungal activity, reducing the problems caused by the trailing effect in the visual endpoints [11, 14, 16]. The agar-based E-test method (AB *Biomerieux*, France) is a commercially available, pre-formed, pre-defined and stable antibiotic method, which uses a plastic test strip impregnated with a continuous concentration gradient of an antifungal agent to determine the minimum inhibitory concentration (MIC) necessary for inhibition of fungal growth [15-17]. It has been proven as a useful method in testing *Candida* spp. against a variety of antifungal agents, including amphotericin B, the azoles and caspofungin [8, 18-20].

The aim of this study was to evaluate the suitability of SYO method for susceptibility profiles' determination of non-*Aspergillus* moulds and of E-test for *Aspergillus* spp. and non-*Aspergillus* moulds, and, where available, other methods. The study was performed at the Laboratory for Medical Mycology at the Institute for Medical Microbiology, University of Zürich, Switzerland.

Material and Methods

Test isolates

In this study, a total of 33 clinical isolates belonging to different species of filamentous fungi from the collection of the mycology laboratory (*Aspergillus fumigatus* (9), *A. glaucus* (2), *A. flavus* (1), *A. niger* (1), *A. nidulans* (1), *A. sclerotiorum* (1) *A. versicolor* (1), *Fusarium* spp. (5), *Paecilomyces variotii* (1), *Paecilomyces* spp. (3), *Scedosporium apiospermum* (5), *Scedosporium prolificans* (2), *Scopulariopsis* spp. (1)), were tested during a fellowship stay at the Laboratory for Medical Mycology at the Institute for Medical Microbiology, University of Zürich, Switzerland in 2006.

Minimum inhibitory concentrations have been determined with Sensititre YeastOne (SYO) method (for *Aspergillus* spp.) and BMD method (for non-*Aspergillus* moulds). Culture and identification of the strains were done by using conventional mycological methods. All isolates were stored in skim milk at –70°C until they were used in the study. They were restored and subsequently passed at least twice on potato carrot agar (non-*Aspergillus* moulds) and malt yeast agar (*Aspergillus* spp.) to ensure viability and adequate sporulation before AFST with E-test (*Aspergillus* spp. and non-*Aspergillus* moulds) and SYO (non-*Aspergillus* moulds) was performed. Inoculum suspensions were prepared from 5-7 days cultures in sterile saline solution with Tween 80 for SYO method as well as for E-test. These suspensions were vortexed for 15 seconds to allow complete uniformity and left for another 15 minutes to allow sedimentation of hyphae. Final inocula were achieved by counting the number of conidia in a Neubauer chamber and were in the range between $0.5x10^6$-$5x10^6$CFU/ml for SYO method and E-test. A confirmatory plate count was done from a 1:1000 dilution to ensure inoculum density on brain heart infusion agar (expected colony forming units (CFU)=15-80; all inocula were in the range between 15-

80 CFU). The quality control (QC) strain *C. parapsilosis* ATCC 22019 (9) was included as control strain for both methods.

M38-A reference broth microdilution method (BMD)

A broth microdilution was performed according to the CLSI document M38-A using colorimetric endpoints. The antifungal drugs used in the study were obtained as reagent-grade powders: amphotericin B (AB) (Sigma Chemical Co.), itraconazole (IZ) (Janseen Pharmaceutica), voriconazole (VZ) (Pfizer). Stock solutions of the antifungal agents were prepared in 100% dimethyl sulfoxide and diluted 100 times to their final concentration, further diluted in RPMI 1640 medium, for all antifungal agents (except for amphotericin B, for which AM3 was used), buffered to pH 7.0 with MOPS buffer, and dispensed into 96-well microdilution trays (Fig. 1). The final concentration of the antifungal agents in the wells ranged from 0.06 to 16 μg/ml (amphotericin B, itraconazole), from 0.03 to 8 μg/ml (voriconazole), and from 0.06 to 16 μg/ml (caspofungin). The inoculated microdilution trays containing Alamar blue were incubated at 35°C and read at 24, 48 and 72 hours. Growth of filamentous fungi was evident as a change in the colorimetric growth indicator from blue (negative) to red (positive). The MIC endpoint was defined as the lowest concentration that produced complete inhibition of growth, i.e., the first blue well (for amphotericin B) or the lowest concentration that substantially inhibited growth, i.e., the first well which changed colour from blue to pink or red (for azoles and caspofungin) [9].

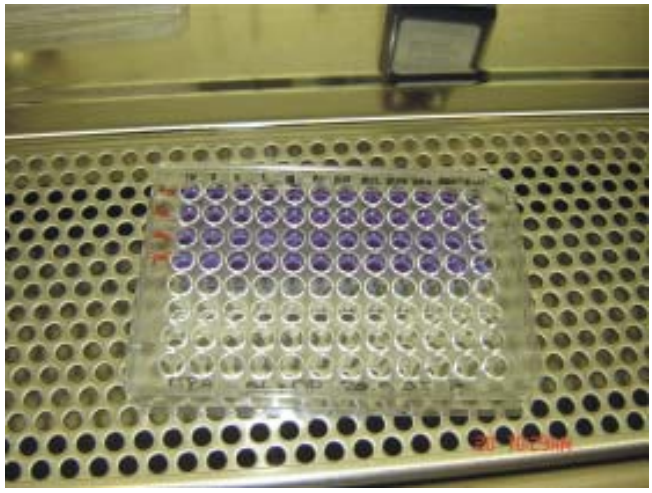

Figure 1: Microdilution trays for the reference broth microdilution method.

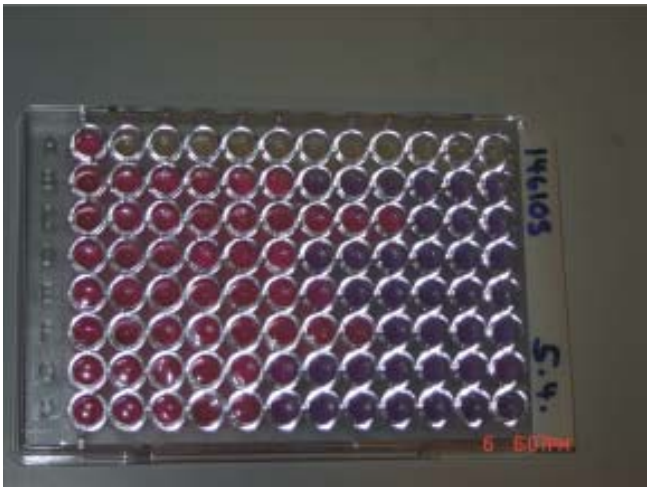

Figure 2: SYO5 Sensititre panels.

Sensititre Yeast One method (SYO method)

SYO5 panels (Trek Diagnostic System, UK) with incorporated Alamar blue containing twofold serial dilutions of dried antifungal drugs were used (Fig. 2). The concentration range for each agent is as follows (Amphotericin B = 0.008-16 μg/ml; Fluconazole = 0.125-256 μg/ml; Itraconazole = 0.008-16 μg/ml; Ketoconazole = 0.008-16 μg/ml; Flucytosine = 0.03-64 μg/ml;

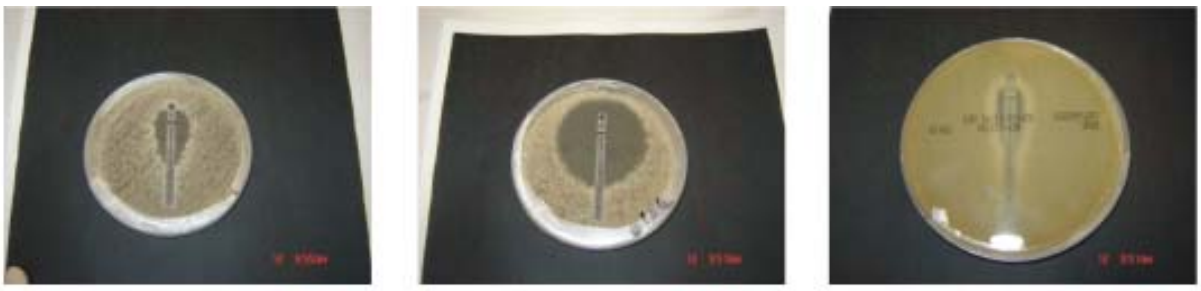

Figure 3: E-test gradient strips of different antifungal agents.

Voriconazole = 0.008-16 μg/ml; Caspofungin = 0.008-16 μg/ml). Inoculum suspensions were diluted 1:100 in YeastOne RPMI medium, the dried SYO panels were rehydrated with 100 ml of the working suspension which was dispensed into each well with a multichannel pippeting device. The panels were covered with seal strips and incubated at 35°C for 24, 48 and 72 hours in a non-CO_2 incubator and then visually read under normal laboratory lighting. Growth of filamentous fungi was evident as a change in the colorimetric growth indicator from blue (negative) to red (positive). The colorimetric MIC was considered to be the lowest concentration of an antifungal agent without any change in the colour (i.e., the first blue well) [11].

E-test method

The E-test method was performed in accordance with the manufacturer's instructions. E-test gradient strips of amphotericin B (AB), itraconazole (IZ), voriconazole (VZ), and caspofungin (CS) were obtained from Axon Lab AG, Baden, Switzerland, posaconazole (PZ) was obtained from Essex Chemie AG, Luzern, Switzerland. The strips were stored frozen on –20°C until they were used in the study. Before AFST, they were left on room temperature for 30 minutes. The agar formulation used for the E-test was RPMI 1640, supplemented with 1.5% agar and 2% glucose and buffered to pH 7.0 with 0.165 M morpholine propanesulfonic acid (MOPS) buffer. The 90-mm-diameter plates contained RPMI at a depth of 4.0 mm. The plates were inoculated by dipping a sterile swab into the cell suspension and streaking it across the surface of the agar in three directions. The plates were dried at ambient temperature for 15 minutes before applying the E-test strips. The plates with the E-test strips were incubated at 35°C and read at 24 or 48 hours for *Aspergillus* spp. (depending on the species growth) and at 48 or 72 hours for other non-*Aspergillus* moulds. The E-test MIC of azoles and caspofungin was considered as the drug concentration at the point where dense colonial growth intersected the strip, ignoring sparse subsurface hyphae at the margins. For amphotericin B, a complete inhibition of growth was read. Microcolonies within the ellipse were ignored [13].

Data analysis

E-test MICs read at 48 h were compared to BMD and SYO MICs read at 48 h. Since the E-test scale has a continuous gradient of concentrations, the MICs with the E-test in between twofold dilutions were rounded to the next higher level for comparison [12-14]. For those strains in which the difference in MICs with the BMD was more than two-fold dilutions, MICs were repeated by SYO and E-test.

Results

The susceptibility profile of different *Aspergillus* spp. to antifungal agents was determined with E-test; results were compared with previous results of SYO

Table 1: In vitro susceptibility of *Aspergillus* spp. a tested by SYO, and E-test methods.

Organism (no. Isolates)	Antifungal	SYO		E-test	
		Range	MIC_{50} [1)]	Range	MIC_{50} [1)]
A. fumigatus (9)	Amphotericin B	0.5-2.0	1.0	0.19-4.0	2.0
	Fluconazole	128-7256	7256	-	-
	Itraconazole	0.032-0.5	0.125	0.19-0.5	0.38
	Ketoconazole	0.5-8.0	2.0	-	-
	Posaconazole	-	-	0.094-0.125	0.094
	Voriconazole	0.064-0.5	0.25	0.064-0.125	0.125
	5-Fluorocytosine	0.5-764	64.0	-	-
	Caspofungin	"		0.047-0.125	0.094
A. glaucus (2)	Amphotericin B	1.0-1.0		0.094-2.0	
	Fluconazole	64-7256	-	-	-
	Itraconazole	0.008-0.125	-	0.004-0.125	-
	Ketoconazole	0.016-4.0	-	-	-
	Posaconazole	-	-	0.004-0.064	-
	Voriconazole	0.064-0.5	-	0.002-0.023	-
	5-Fluorocytosine	8.0-32.0	-	-	-
	Caspofungin	"		0.044-0.125	
A. flavus (1)	Amphotericin B	2.0		6.0	
	Fluconazole	>256	-	-	-
	Itraconazole	0.125	-	0.25	-
	Ketoconazole	1.0	-	-	-
	Posaconazole	-	-	0.25	-
	Voriconazole	0.125	-	0.25	-
	5-Fluorocytosine	2.0	-	-	-
	Caspofungin	"		0.16	
A. sclerotiorum (1)	Amphotericin B	4.0		4.0	
	Fluconazole	>256	-	-	-
	Itraconazole	0.5	-	0.5	-
	Ketoconazole	4.0	-	-	-
	Posaconazole	-	-	0.125	-
	Voriconazole	0.25	-	0.125	-
	5-Fluorocytosine	>64	-	-	-
	Caspofungin			0.094	
A. niger (1)	Amphotericin B	1.0		0.5	
	Fluconazole	>256	-	-	-
	Itraconazole	0.5	-	2.0	-
	Ketoconazole	4.0	-	-	-
	Posaconazole		-	0.25	-
	Voriconazole	0.5	-	0.38	-
	5-Fluorocytosine	64.0	-	-	-
	Caspofungin	"		0.032	
A. nidulans (1)	Amphotericin B	2.0	-	1.0	-
	Fluconazole	128	-	-	-
	Itraconazole	0.125	-	0.125	-
	Ketoconazole	0.125	-	-	-
	Posaconazole	.		0.125	
	Voriconazole	0.064	-	0.125	-
	5-Fluorocytosine	>64	-	-	-
	Caspofungin			>16	
A. versicolor (1)	Amphotericin B	4.0		4.0	
	Fluconazole	>256	-	-	-
	Itraconazole	0.25	-	0.25	-
	Ketoconazole	0.5	-	-	-
	Posaconazole	-	-	0.094	-
	Voriconazole	0.125	-	0.125	-
	5-Fluorocytosine	>64	-	-	-
	Caspofungin	-	-	0.094	-

-, Not determined; 1) MIC_{50} (MIC causing inhibition of 50% of isolates) values were calculated for those species with 3 or more isolates.

method. For non-*Aspergillus* moulds SYO and E-test were performed, and results were compared with previous results of CLSI BMD method. All strains produced detectable growth after 48h of incubation, except one strain of *A. glaucus* and one strain of *Paecilomyces* spp. that required 6 and 7 days of incubation respectively for growth in SYO plates. The susceptibility testing was performed twice for these strains to ensure avoidance of technical errors. Results with control strain were within the described range according to reference documents [9]. E-test inhibition ellipses were clear. Triazole ellipses, especially those of voriconazole were wider than amphotericin B ellipses for most isolates. Tables 1 and 2 summarize the antifungal susceptibility profiles of the 33 strains of *Aspergillus* spp. and non-*Aspergillus* moulds to amphotericin B, itraconazole, voriconazole, posaconazole and caspofungin obtained with the E-test, to amphotericin B, fluconazole, itraconazole, ketoconazole, flucytosine and voriconazole as determined by the SYO method and to amphotericin B, itraconazole, voriconazole and caspofungin as determined with the CLSI BMD.

Table 2: In vitro susceptibility of non-*Aspergillus* moulds a tested by SYO, E-test, and BMD methods.

Organism (no. Isolates)	Antifungal	SYO		E-test		BMD	
		Range	MIC_{50} [1]	Range	MIC_{50} [1]	Range	MIC_{50} [1]
Fusarium sp. (5)	Amphotericin B	0.25-2.0	1.0	0.25-4.0	1.5	2.0-8.0	4.0
	Fluconazole	≥256-≥256	≥256	-	-	-	-
	Itraconazole	≥16-≥16	≥16	≥32-≥32	≥32	16-≥16	≥16
	Ketoconazole	8.0-≥16	≥16	-	-	-	-
	Posaconazole	-	-	8.0-≥32	≥32	-	-
	Voriconazole	1.0-4.0	4.0	2.0-4.0	4.0	4.0-≥8.0	4.0
	5-Fluorocytosine	≥64-≥64	≥64	-	-	-	-
	Caspofungin	-	-	≥32-≥32	≥32	0.12-≥16	4.0
Scedosporium apiospermum (5)	Amphotericin B	2.0-≥16	2.0	4.0-≥32	32	4.0-≥16	≥16
	Fluconazole	16-32	16	-	-	-	-
	Itraconazole	0.064-0.5	0.5	0.25-2.0	0.5	0.5-2.0	1.0
	Ketoconazole	0.125-0.5	0.25	-	-	-	-
	Posaconazole	-	-	0.25-2.0	0.5	-	-
	Voriconazole	0.064-0.125	0.125	0.064-0.64	0.125	0.12-0.25	0.25
	5-Fluorocytosine	≥64-≥64	≥64	-	-	-	-
	Caspofungin	-	-	≥32-≥32	≥32	-	-
Scedosporium prolificans (2)	Amphotericin B	4.0-≥16	-	≥32-≥32	-	16-≥16	-
	Fluconazole	≥256-≥256	-	-	-	-	-
	Itraconazole	≥16-≥16	-	≥32-≥32	-	-	-
	Ketoconazole	4.0-≥16	-	-	-	-	-
	Posaconazole	-	-	0.25-≥32	-	-	-
	Voriconazole	2.0-16	-	1.5-≥24	-	1.0-2.0	-
	5-Fluorocytosine	≥64-≥64	-	-	-	-	-
	Caspofungin	-	-	≥32-≥32	-	-	-
Paecilomyces sp. (3)	Amphotericin B	2.0-≥16	2.0	2.0-≥32	≥32	8.0-≥16	≥16
	Fluconazole	32-≥256	≥256	-	-	-	-
	Itraconazole	1.0-≥16	≥16[2]	0.75-≥32	≥32	8.0-≥16	≥16
	Ketoconazole	2.0-≥16	-	-	-	-	-
	Posaconazole	-	-	1.0-2.0	2.0	-	-
	Voriconazole	0.25-≥16	1.0[2]	0.125-≥32	0.64	0.5-1.0	1.0
	5-Fluorocytosine	0.03-2.0	-	-	-	-	-
	Caspofungin	-	-	0.16-≥32	≥32	-	-
Paecilomyces varioti (1)	Amphotericin B	0.5	-	0.125	-	0.5	-
	Fluconazole	≥256	-	-	-	-	-
	Itraconazole	0.064	-	0.094	-	0.5	-
	Ketoconazole	1.0	-	-	-	-	-
	Posaconazole	-	-	0.094	-	-	-
	Voriconazole	8.0	-	8.0	-	8.0	-
	5-Fluorocytosine	0.25	-	-	-	-	-
	Caspofungin	-	-	0.012	-	-	-
Scopulariopsis sp. (1)	Amphotericin B	4.0	-	≥16	-	≥16	-
	Fluconazole	≥256	-	-	-	-	-
	Itraconazole	≥16	-	≥32	-	≥16	-
	Ketoconazole	0.5	-	-	-	-	-
	Posaconazole	-	-	≥32	-	-	-
	Voriconazole	4.0	-	≥32	-	8.0	-
	5-Fluorocytosine	≥64	-	-	-	-	-
	Caspofungin	-	-	24	-	-	-

-, Not determined; 1) MIC_{50} (MIC causing inhibition of 50% of isolates) values were calculated for those species with 3 or more isolates; 2) MIC_{50} was not determined because 1 of 3 values was not determined.

SYO versus E-test

In 9/33 (27%) mould isolates amphotericin B MICs were found to be identical by SYO and E-test, in 12/33 (36%) isolates amphotericin B MICs differed by ±1 dilution, and in 7 isolates (21%) they differed by ±2 dilutions (Table 3). Differences were most frequently due to higher MICs with the E-test. Discrepancies (greater than ± 2 dilutions) between the MICs determined by the SYO method and E-test were demonstrated in 5/33 (15%) isolates tested against amphotericin B: *1 A. glaucus*, 1 *Scedosporium prolificans*, 2 *Scedosporium apiospermum*, and 1 *Paecilomyces* spp., and these discrepancies were almost always due to higher MICs with the E-test (except for *A. glaucus*, for which E-test result was lower). Overall, 4 strains were classified as amphotericin B ''resistant" (MIC>32 μg/ml) by the Etest, but SYO method failed to detect these strains. In 16 out of 33 (48%) moulds itraconazole MICs were determined as identical by both methods, in 11/33 (33%) isolates the itraconazole MICs differed by ± 1 dilution with E-test, and in 4/33 (12%) the itraconazole MICs were two dilutions higher by the E-test. Two (6%) major discrepancies between the itraconazole MICs with both methods were observed. For voriconazole, in 11/33 (33%) voriconazole MICs were found to be identical by both methods, in 13/33 (39%) isolates the voriconazole MICs differed by ±1 dilution. In 4/33 (12%) the voriconazole MICs differed by two dilutions. In 5 (15%) strains major discrepancies with more than 2 dilution higher values in favour of E-test were registered.

SYO versus M-BMD

In 4/17 (24%) non-*Aspergillus* moulds, amphotericin B MICs were determined to be identical by SYO method and BMD, in 3/17 (18%) isolates amphotericin B MICs differed by one dilution, and in 6/17 (35%) MICs for amphotericin B differed by two dilutions higher. Where differences by one or two dilutions were observed, they were due to higher MICs with BMD. In 4 (24%) strains, major discrepancies with more than 2 dilutions higher values occurred and they were due to higher MICs with BMD. In 4/17 (24%) isolates of non-*Aspergillus* moulds, the itraconazole MICs were identical by both methods, in 3/17 (18%) isolates differed by ±1 dilution, and in 1 (6%) isolate by two dilutions. In 4 (24%)

strains, major discrepancies with more than 2 dilutions were noticed. In 5/17 (29%) the MIC with BMD was not determined (data not shown). In 4/17 (24%) isolates of non-*Aspergillus* moulds, the voriconazole MICs were determined identical by both methods, in 10/17 (59%) isolates they differed by ±1 dilution, and in one (6%) isolate by two dilutions. In 2 (12%) strains, major discrepancies with more than 2 dilutions were noticed.

Table 3: Agreement between SYO and E-test, BMD and SYO and M-BMD and E-test for three agents tested against 33 isolates of *Aspergillus* spp. and non- *Aspergillus* moulds.

Agreement between methods	Antifungal agent	% Agreement	
		± 1 dilution	± 2 dilutions
SYO and E-test (n = 33)	Amphotericin B	63.6	84.9
	Itraconazole	81.8	93.9
	Voriconazole	72.7	84.8
BMD and SYO (n = 17)	Amphotericin B	41.2	76.5
	Itraconazole	58.3	66.7
	Voriconazole	82.4	88.2
BMD and E-test (n = 17)	Amphotericin B	47.1	82.4
	Itraconazole	58.3	83.3
	Voriconazole	64.7	82.4

E-test versus MBD

In 7/17 (41%) isolates of non-*Aspergillus* moulds, amphotericin B MICs were determined to be same by both methods, in 1 (6%) isolate amphotericin B MIC differed by ±1, and in 6/17 (35%) amphotericin B MIC differed by ±2 dilutions. Discrepancies (greater than ±2 dilutions) between the MICs determined by BMD and Etest were demonstrated in 3/17 (18% tested against amphotericin B. In 6/17 (35%) isolates of non-*Aspergillus* moulds, itraconazole MICs were determined to be identical by both methods, in 1 (6%) isolate itraconazole MIC differed by ±1 dilution, and in 3/17 (18%) isolates they differed by ±2 dilutions. Discrepancies (greater than ±2 dilutions) between the MICs determined by the BMD and E-test were demonstrated in 2/17 (12%) tested against itraconazole. In 5/17 (29%) the MIC with the BMD was not determined (data not shown). In 4/17 (24%) isolates of non-*Aspergillus* moulds, voriconazole MICs were determined to be identical by both methods, in 7/17 (41%) isolates voriconazole MICs differed by ±1 dilution, and in 3 (18%) isolates voriconazole MIC differed by ±2 dilutions. Major discrepancies (greater than ±2 dilutions) between the MICs determined by BMD and Etest were demonstrated in 3/17 (18%) tested against voriconazole (always due to higher MICs with E-test).

The MIC results obtained by both BMD and E-test methods demonstrated that voriconazole was very active against this diverse array (5 different genera) of opportunistic non-*Aspergillus* moulds (69.7% susceptible at MIC <1 μg/ml). Elevated MICs as determined by all methods were more frequently observed with *Fusarium* spp., as well as with isolates of *Scopulariopsis* spp. and *Paecilomyces* spp. When discrepancies between the BMD and E-test MICs were noted, the E-test tended to give lower values with voriconazole. All isolates of *Aspergillus* spp. were inhibited with <0.5 μg/ml of voriconazole. These results demonstrate the excellent efficacy of voriconazole against *Aspergillus* species and suggest that voriconazole may be the treatment of choice in invasive aspergillosis caused by *A.fumigatus* and *A.flavus*. Indeed, this is supported by clinical studies, which have shown better responses to voriconazole than to amphotericin B in patients with invasive aspergillosis (21-24).

Discussion

We found the E-test to be a very simple method for determination of susceptibility profile of filamentous moulds to all antifungals, except of few difficulties experienced with caspofungin, where residual growth was noticed. RPMI agar with glucose (final concentration, 2%) supported optimal growth of all species tested and provided good agreement with the MICs obtained with BMD. Szekely et al. [25] used RPMI agar and 48 h of incubation and found that the inhibition ellipses were clear for most isolates. They concluded that the E-test procedure was reproducible and served as a suitable method for AFST of moulds. Pfaller et al. demonstrated that the E-test was able to detect resistance to itraconazole among filamentous fungi [15]. Additionally, the depth of the agar can influence the MIC. Therefore, the manufacturer's recommendations should be followed when attempting to obtain MICs by E-test. The SYO method has the advantage of being technically easy to perform and able to determine objective and quantitative MIC endpoints [27]. The BMD method is regarded as the reference method, however, a main disadvantage of this procedure is that the preparation of the trays is time consuming and laborious.

The results of this study show that the overall agreement at ±2 dilutions between the three methods is relatively high. However, the correlation of the SYO method to the BMD method is only moderate for amphotericin B and itraconazole. The vast majority of

differing results are due to higher MICs with the BMD method. In particular, SYO was unable to detect some of the potentially amphotericin B resistant strains. Likewise, when comparing the results of the SYO method to the Etest method for amphotericin B and itraconazole, the majority of differing results are in favour of higher MICs with the E-test. Similarly, Meletiadis et al. [14] have shown lower SYO MICs for itraconazole in several *Aspergillus* spp. (except *A. ustus*). They could not show this effect for amphotericin B. In contrast, in another study in which also E-test, SYO and BMD were compared [27], MIC ranges by E-test were broader and always higher for amphotericin B and itraconazole.

The MIC results obtained by E-test methods demonstrated that posaconazole was very active against all *Aspergillus* spp. (all susceptible at MIC <0.25 μg/ml), but showed poor activity against some of the non-*spergillus* moulds (*Fusarium* spp., *Scedosporium* spp., *Scopulariopsis* spp.) (28, 29). SYO method includes fluconazole and 5-flucytosine, to which in general filamentous fungi are resistant and are not useful for therapy (panel testing thus generates more costs). Also, with SYO it is not possible to test caspofungin for moulds, because a majority of the strains appear resistant, whereas E-test allows testing of antifungal drugs according to the needs of the clinician (including caspofungin and posaconazole). However, SYO has potential value for the performance of susceptibility testing of filamentous fungi to other antifungal agents, such as itraconazole and voriconazole, so it could be a good and reliable alternative for in vitro AFST, especially with SYO8 which includes the newer triazole, posaconazole. But the results provided with this study indicate that SYO is not sensitive enough for in vitro detection of resistance to amphotericin B in *Aspergillus* spp. and non-*Aspergillus* moulds.

In conclusion, on the basis of data from this study and other studies as well, the ability of E-test to generate MIC data for filamentous fungi that are comparable to those obtained by the CLSI broth microdilution method has been shown and thus a potential value for E-test for the use for AFST of mould pathogens has been proved. This could be attractive to microbiology laboratories, since it will provide the flexibility to test one or more commonly used antifungal agents selectively against a wide variety of moulds that may be encountered clinically. The higher amphotericin B MICs obtained by the E-test as compared to SYO for some strains suggests that this method could be more useful in detection of mould isolates potentially resistant to amphotericin B. In general, both the E-test and the SYO methods are promising, but require further investigation to identify the optimum conditions for their use in the testing of the susceptibilities of filamentous fungi to antifungal agents, including new azoles and the new class of echinocandins.

Optimization of these tests might require adjustments depending on the species tested. However, more studies are required to determine which methods show the best agreement with in vivo results, and thus, the clinical value of these in vitro results should be determined in clinical trials.

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