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ETEST FOR ASSESSING THE SUSCEPTIBILITY OF FILAMENTOUS FUNGI*

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*Dedicated to the 100th anniversary of the birth of Professor György Ivánovics

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Abstract

The purpose of this study was to evaluate the Etest as an *in vitro* antifungal susceptibility test method for different moulds originating from human samples and from the environment. A total of 50 isolates (1 *Acremonium*, 18 *Aspergillus*, 2 *Cladosporium*, 1 *Epicoccum*, 15 *Penicillium*, 2 *Scopulariopsis* and 11 *Trichoderma* strains) were tested by the Etest. 46 of the tested moulds (92%) were resistant to fluconazole with minimal inhibitory concentrations (MICs) $\geq$ 256 μg ml^{-1}. There were strains resistant to itraconazole among *Aspergillus niger*, *Aspergillus ochraceus* and *Cladosporium* spp. with MICs > 32 μg ml^{-1}. For fluconazole, no differences were observed using two different inocula, while for itraconazole, ketoconazole and amphotericin B, a 1 or less step 2-fold dilution difference in MIC was seen for the most of 10 selected strains. The MICs of fluconazole and amphotericin B obtained for *Trichoderma* strains by the Etest and the agar dilution method were also compared. MICs for fluconazole were in agreement, while MICs for amphotericin B were higher with 1 or 2 steps of 2-fold dilutions for most of *Trichoderma* strains in the case of the agar dilution method.

Introduction

The incidence of fungal infections has been increasing since the 1980s. The increasing number of susceptible hosts (organ or bone marrow transplant patients) as well as the use of immunsuppressive agents and antimicrobial prophylactic strategies has probably contributed to the changing epidemiology of mycoses [1]. The volume of disseminated infections caused by filamentous fungi (moulds) is lower than that caused by yeasts [2]. Most infections caused by moulds originate from the environment by inhalation through the respiratory tract or contamination (e.g. postoperative wounds, heart valves, etc.) [3]. The most frequently isolated moulds are *Aspergillus* spp.: *A. fumigatus* is responsible for a large majority (85-90%) of the different clinical manifestations of several mould infections [2]. However, the importance of other moulds (*Penicillium*, *Cladosporium* and *Scopulariopsis* spp.) has also been increasing. Moulds are able to cause infections of the respiratory tract (e.g. pneumonia, allergic bronchopulmonary mycosis, aspergilloma, allergic fungal rhinosinusitis, etc.) and superficial, cutaneous, osseous or invasive mould infections [4–10].

The *in vitro* susceptibility testing of filamentous fungi is becoming increasingly important because of the frequency and diversity of the infections they cause [11]. The standard method for determination of the MICs of filamentous fungi (the NCCLS broth microdilution method [12, 13]) and the Etest were compared in previous studies [2, 14, 15]. In the case of many moulds, good correlations have been demonstrated for amphotericin B and itraconazole, but the results depended on the species tested and the nutrient media used for susceptibility testing [2, 14].

This report summarizes the antifungal susceptibility results on filamentous fungi isolated from clinical specimens and from the environment, obtained by the Etest method.

Materials and methods

Isolates

A total number of 50 isolates were tested, including 44 clinical isolates obtained from different specimens (from the upper or lower respiratory tract, the gastrointestinal tract, the genital tract or the peritoneal fluid as well as mucocutaneous samples). Strains were isolated from Sabouraud chloramphenicol agar (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) and maintained on Sabouraud chloramphenicol agar slants until use in this study. The clinical isolates included 7 *Trichoderma* strains obtained from the American Type Culture Collection (Manassas, USA) and the University of Alberta Microfungus Collection and Herbarium, these strains originated from immunosuppressed patients with various infections. Among 6 strains originating from the environment, 4 *Trichoderma* isolates were studied earlier for cold tolerance [16].

All isolates were subcultured on Sabouraud chloramphenicol agar plates and incubated at 30 °C until adequate growth was attained before their use in susceptibility testing.

Etest for MIC determinations

The Etest was performed according to the manufacturer's instructions (AB Biodisk; Solna, Sweden) [17]. Spore suspensions were adjusted to match the 0.5 McFarland standard. The medium used was RPMI 1640 agar (15 g l^{-1}) supplemented with 20 g l^{-1} glucose. This medium was dispensed in 20 ml amounts into Petri dishes (90 mm in diameter) giving an agar depth of 4 ± 0.5 mm. These plates were inoculated by dipping a sterile swab into the inoculum suspension and streaking it across the entire surface in three directions. The plates were dried at room temperature for 15 min before the application of the Etest strips. (The drug

concentrations ranged from 0.016 to 256 mg l^{-1} for fluconazole, and from 0.002 to 32 mg l^{-1} for itraconazole, ketoconazole and amphotericin B). The plates were incubated at 30 °C and read after incubation for 24, 48 or 72 hours according to the growth conditions of the isolates. The MIC was read as the drug concentration at which the elliptical inhibition zone intersected the scale on the antifungal test strip.

Agar dilution method

The susceptibility of *Trichoderma* strains was evaluated for fluconazole and amphotericin B by the agar dilution method as well [18]. Stock solutions of 2 mg ml^{-1} were prepared from amphotericin B (Bristol-Myers Squibb AG, Baar) and fluconazole (Pfizer, Amboise, France) in sterile distilled water. 20 ml aliquots of RPMI 1640 agar (15 g l^{-1}) supplemented with 20 g l^{-1} glucose were used to fill the 90-mm-diameter Petri dishes. Appropriate quantities of the stock solutions were added to the molten and cooled medium to reach final concentrations of 4–512 μg ml^{-1} for fluconazole and 0.25–32 μg ml^{-1} for amphotericin B, corresponding to the Etest concentrations. The medium was mixed and allowed to solidify undisturbed. Control plates containing the test medium without antimicrobial agents were prepared for each strain.

3-mm-diameter discs were cut from the edges of young colonies of *Trichoderma* strains [16]. One disc was inoculated onto each plate, and the plates were then incubated at 30 °C for 48 hours. The MIC was read as the lowest drug concentration at which no growth of the microorganisms was detected.

Results

During the past years (2000-2002), 50 filamentous fungi originating from different specimens were evaluated. Table I shows the origin and distribution of the isolates. No parallel isolates from the same patient were involved in the study.

32 human isolates were cultured from specimens from the upper or lower respiratory tract: mucin from the nose, washing fluid from the paranasal sinuses or nose, specimens from the ears or thoracic fluid. 3 isolates were from the peritoneal fluid, 4 from the gastrointestinal tract, 3 from mucocutaneous samples and 2 from the genital tract. The most frequently isolated species were *A. versicolor* among the *Aspergillus* spp. and *P. chrysogenum* among the *Penicillium* spp. The 6 isolates from environmental samples were *Epicoccum nigrum* (1), *Scopulariopsis* spp. (1), *T. citrinoviride* (1), *T. koningii* (1), *T. longibrachiatum* (1) and *T. pseudokoningii* (1).

These fungi were tested and evaluated for their susceptibilities to fluconazole, ketoconazole, itraconazole and amphotericin B by the Etest (Table II). 46 of the tested moulds (92%) were resistant to fluconazole, with MIC >256 μg ml^{-1}. The range of MICs for ketoconazole was wide: there were resistant strains with MICs >32 μg ml^{-1} among the *A. niger*, *A. ochraceus* and *Cladosporium* spp., while the lowest MIC values were obtained for the *Trichoderma* strains (0.008–0.5 μg ml^{-1}). Two strains were resistant to amphotericin B with MIC values >32 μg ml^{-1}.

For 10 selected isolates, the MICs obtained by the Etest were measured by using two different spore suspension turbidities. The MICs obtained after incubation for 48 or 72 hours are listed in Table III. For fluconazole, no differences were observed with the two inocula: all strains were fully resistant. For itraconazole, ketoconazole and amphotericin B, a 1- or 2-step 2-fold dilution difference in MIC was seen for most strains with the exception of *A. niger* 2.

and *A. ochraceus*: significant differences in MICs were detected for ketoconazole and itraconazole in the case of these two strains.

The MICs of fluconazole and amphotericin B obtained for 10 *Trichoderma* strains by the Etest and the agar dilution method were compared. Table IV presents these results together with data from appropriate references. The MICs of fluconazole obtained for 8 strains with the two methods were in agreement, but higher values were obtained by the Etest for strain *T. koningii* T 39. Higher MICs were obtained for amphotericin B with the agar dilution method than with the Etest in the case of 7 strains.

Discussion

Most of the infections caused by moulds originate from the environment. These fungi pass into the human body through breathing, food or contamination. During the past two years, moulds were isolated most frequently from the upper respiratory tract. Predominantly these areas (nose, paranasal sinuses and ears) are exposed to fungal spores. Isolates from the gastrointestinal tract originated from contaminated food: most of these fungi are evacuated from the organism, but they may be the origin of serious mould infections in immunosuppressed patients [20]. The rates of *Aspergillus* spp. (18 isolates) and *Penicillium* spp. (15 isolates) in human specimens during the 2-year period were similar. *Acremonium* spp., *Cladosporium* spp. and *Scopulariopsis* spp. were isolated in small numbers.

There have been previous reports on comparisons of antifungal susceptibility testing methods for moulds [2, 14, 15], and a good correlation was observed between the Etest and broth microdilution methods. In this present study, most strains were resistant to fluconazole by the Etest (Table II), while ketoconazole, itraconazole and amphotericin B were more effective. For most strains, the MICs were not influenced significantly by the turbidity of spore suspension (Table III). For 8 of 10 strains, there were no or only small differences between the MICs obtained on the use of 3 McFarland and 0.5 McFarland suspensions (in accordance with the manufacturer's instruction). If the higher inoculum was used for some slow-growing isolates, the MICs could be read 1 day earlier. Higher differences were detected for 2 *Aspergillus* strains: *A. niger* and *A. ochraceus*. The MICs of these strains for amphotericin B and fluconazole did not differ markedly, and were similar to the itraconazole MIC of *A. ochraceus*. These moulds grew rapidly and covered the surface of the agar plates completely (with the exception of the inhibition zone) when a higher turbidity of suspension was used. The cultures were thicker, which influenced the MICs. More exact values could be

obtained if the turbidity of the spore suspension was measured spectrophotometrically in the case of black moulds.

The differences in MIC values obtained by comparison of the Etest and the agar dilution methods were similar for the evaluated *Trichoderma* strains (Table IV). The MICs for fluconazole did not differ markedly with these methods, but they did differ by 1 or 2 steps of 2-fold dilutions from the data in the literature obtained by broth dilution methods. The MICs for amphotericin B were higher by 1 or 2 steps of 2-fold dilutions with the agar dilution method.

In conclusion, these data indicated differences between the susceptibility testing methods for moulds. The agar dilution method was not the most appropriate for the susceptibility testing of moulds in routine laboratory use, because the exact results depended on numerous features (e.g. the stability of the solution of antimycotics, the mixing of the stock solutions into the agar medium, the growth rate of the moulds, etc.). The Etest is easier to perform: it is less labour-intensive and much simpler to set up than the broth microdilution or agar dilution methods [2, 15] and it provides the flexibility to test antifungal agents against different moulds [14].

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Table I

Origin of moulds evaluated for antifungal susceptibility

Species	No. of isolates						Total No. of isolates (% of all)
	Gastrointestinal tract	Upper and lower respiratory tract	Peritoneal fluid	Mucocutaneous samples	Genital tract	Environmental samples	
Acremonium spp.	0	1	0	0	0	0	1 (2%)
A. candidus	0	1	0	0	0	0	1 (2%)
A. fumigatus	0	4	0	0	0	0	4 (8%)
A. niger	0	4	0	1	0	0	5 (10%)
A. ochraceus	0	2	0	0	0	0	2 (4%)
A. versicolor	0	3	0	1	2	0	6 (12%)
Cladosporium spp.	0	2	0	0	0	0	2 (4%)
E. nigrum	0	0	0	0	0	1	1 (2%)
P. chrysogenum	0	6	0	0	0	0	6 (12%)
P. humicola	2	0	0	0	0	0	2 (4%)
P. humuli	0	4	0	0	0	0	4 (8%)
Penicillium spp.	0	3	0	0	0	0	3 (6%)
Scopulariopsis spp.	1	0	0	0	0	1	2 (4%)
T. citrinoviride	0	0	1	0	0	1	2 (4%)
T. koningii	0	0	1	0	0	1	2 (4%)
T. longibrachiatum	1	2	1	1	0	1	6 (12%)
T. pseudokoningii	0	0	0	0	0	1	1 (2%)
Total	4	32	3	3	2	6	50 (100%)

Table II

Antifungal susceptibilities of moulds evaluated by the Etest method

Species (No. of tested isolates)	Incubation time (h)	(Range of) MICs (μg ml^{-1})			
		Fluconazole	Ketoconazole	Itraconazole	Amphotericin B
Acremonium spp. (1)	72	>256	0.5	>32	2
A. candidus (1)	72	>256	0.032	0.004	8
A. fumigatus (4)	48	>256	0.125 – 4	0.25 – 1	0.016 – 2
A. niger (5)	24, 48	8 - >256	0.016 – >32	0.032 – 4	0.25 – 1
A. ochraceus (2)	48	>256	2 and >32	1 and 4	8 and >32
A. versicolor (6)	48, 72	>256	0.064 – 0.5	0.125 – 2	0.032 – 16
Cladosporium spp. (2)	48, 72	>256	0.064 and >32	0.5 and 1	0.032 and 2
E. nigrum (1)	72	>256	0.016	0.016	0.125
P. chrysogenum (6)	48, 72	>256	0.25 – 1	0.125 - >32	4 – >32
P. humicola (2)	48	>256	0.25 and 0.5	8 and >32	8
P. humuli (4)	48, 72	64 and >256	0.25 – 1	0.064 – 2	0.125 – 1
Penicillium spp. (3)	48, 72	>256	0.125 – 0.5	1 – 2	0.25 – 8
Scopulariopsis spp. (2)	48	>256	0.125 and 1	1 and >32	0.5 and 1
T. citrinoviride (2)	48	>256	0.25 and 0.5	24	0.064 and 2
T. koningii (2)	48	>256	0.25	2 and 4	0.5 and 8
T. longibrachitaum (6)	48	8 - >256	0.008 – 0.5	1 – 32	1 – 2
T. pseudokoningii (1)	48	>256	0.25	8	0.25

Table III

Influence of turbidity of spore suspension on MICs

Species	McFarland	Incubation time (h)	MICs (μg ml^{-1})			
			Fluconazole	Ketoconazole	Itraconazole	Amphotericin B
A. fumigatus 1.	0.5	48	>256	2	1	0.25
	3	48	>256	4	1	0.5
A. fumigatus 2.	0.5	48	>256	4	0.25	0.016
	3	48	>256	4	0.25	0.016
A. niger 1.	0.5	48	>256	2	2	0.25
	3	48	>256	2	2	0.25
A. niger 2.	0.5	48	>256	0.5	4	0.25
	3	48	>256	>32	32	0.5
A. ochraceus	0.5	48	>256	2	1	>32
	3	48	>256	>32	2	>32
A. versicolor	0.5	72	>256	0.125	1	0.25
	3	72	>256	0.25	1	0.5
P. humuli	0.5	72	>256	0.5	2	1
	3	48	>256	0.5	2	1
Penicillium spp.	0.5	72	>256	0.5	2	8
	3	48	>256	0.5	2	8
Scopulariopsis spp.	0.5	48	>256	1	1	0.5
	3	48	>256	2	1	0.5
S. brevicaulis	0.5	72	>256	0.125	>32	1
	3	48	>256	0.25	>32	1

Table IV

MIC values obtained by Etest and agar dilution method for *Trichoderma* spp.

Species	Identity	MIC values (µg ml^{-1})			
		Fluconazole		Amphotericin B	
		Etest	Agar dilution	Etest	Agar dilution
T. citrinoviride	UAMH 9573	64	64	2	2
T. koningii	T 39	>256	64	0.5	2
T. koningii	CM 382 [a]	>256	512	8	16
T. longibrachiatum	TK 51	>256	256	2	4
T. longibrachiatum	UAMH 7955	>256	256	1	4
T. longibrachiatum	UAMH 7956 [b]	64	64	2	8
T. longibrachiatum	UAMH 9515	>256	512	2	4
T. longibrachiatum	ATCC 201044 [c]	64	32	2	2
T. longibrachiatum	ATCC 208859	>256	256	2	4
T. pseudokoningii	T 29	>256	256	0.25	0.25

[a] Ref. 19: MICs : fluconazole: 128 µg ml^{-1}; amphotericin B: 4 µg ml^{-1} (by broth microdilution method)

[b] Ref. 20: MICs: fluconazole: 16 µg ml^{-1}; amphotericin B: 2 µg ml^{-1} (by broth microdilution method)

[c] Ref. 21: MICs: fluconazole: ?64 µg ml^{-1}; amphotericin B: 2 µg ml^{-1} (by broth macrodilution method)