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Susceptibility of clinical isolates of *Fusarium* to antifungal drugs

Antimykotika-Empfindlichkeit klinischer *Fusarium*-Isolate

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Schlüsselwörter. *Fusarium*, Empfindlichkeitsprüfung, Antimykotika, Amphotericin B, Azole, Terbinafin.

Summary. The inhibitory activities of amphotericin B, fluconazole, itraconazole, miconazole, ketoconazole and terbinafine against nine isolates from clinically apparent infections of *Fusarium solani*, four isolates of *Fusarium moniliforme* and 10 isolates of *Fusarium oxysporum* were determined with an agar diffusion method (Neo-sensitabs) and an agar dilution method. The inhibition zones obtained with antifungal Neo-sensitabs need very careful interpretation. We did not find a good correlation between the agar diffusion method using Neo-sensitabs preloaded with azoles and amphotericin B and the agar dilution method. Amphotericin B (12/23) and terbinafine (18/23) showed good activity. Miconazole (7/23) and ketoconazole (3/23) had poor inhibitory activity. Fluconazole and itraconazole (0/23) had no *in vitro* activity against any of the isolates tested.

Zusammenfassung. Die inhibitorische Aktivität von Amphotericin B, Fluconazol, Itraconazol, Miconazol, Ketoconazol und Terbinafin gegen 9 klinische Isolate von *Fusarium solani*, 4 von *Fusarium moniliforme* und 10 von *Fusarium oxysporum* wurde mit einer Agar-Diffusionstechnik (Neo-sensitabs) und einer Agar-Dilutionsmethode untersucht. Die Hemmhöfe mit Neo-sensitabs müssen mit Vorsicht interpretiert werden. Es wurde keine gute Korrelation zwischen der Agar-Diffusionstechnik mit Neo-sensitabs, beladen mit Azolen und Amphotericin B, und der Agar-Dilutionstechnik gefunden. Amphotericin B (12/23) und Terbinafin (18/23) zeigen eine gute Aktivität. Miconazol (7/23) und Ketoconazol (3/23) haben nur schwache inhibitorische Aktivität. Fluconazol und Itraconazol (0/23) zeigen *in vitro* keine Aktivität gegen die untersuchten *Fusarium*-Stämme.

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Introduction

Before 1973 *Fusarium* spp. were frequently considered as contaminants. Since 1973, when Cho *et al.* [1] reported the first case of a disseminated infection in an immunocompromised child, invasive infections due to *Fusarium* spp. have become a growing problem in immunocompromised hosts. Amphotericin B is the most commonly used treatment, sometimes together with 5-fluorocytosine, rifampicin, azoles or granulocyte(–macrophage) colony-stimulating factor (GM-CSF, G-CSF) [2].

We evaluated the inhibitory activities of amphotericin B, fluconazole, itraconazole, miconazole, ketoconazole and terbinafine against 23 clinical isolates of *Fusarium* spp. using an agar diffusion method and an agar dilution method.

Materials and methods

Agar diffusion method

Neo-sensitabs (Rosco Diagnostica, Taastrup, Denmark) preloaded with a constant amount of diffusible antifungal, [amphotericin B (10 μg), fluconazole (15 μg), itraconazole (10 μg), ketoconazole (15 μg), miconazole (10 μg), terbinafine (30 μg) and 5-fluorocytosine (10 μg)] were used in the agar diffusion method. The tablets were placed

on casitone medium (formula Sanofi Pasteur), except the 5-fluorocytosine discs, which were placed on modified Shadomy agar [3].

Agar dilution method

Twofold serial dilutions of all antifungals were made in casitone medium. Amphotericin B (fungizone ad perfusionem) was obtained from Bristol Myers Squibb (Princeton, NJ, USA); fluconazole from Pfizer (New York, NY., USA.); itraconazole, ketoconazole and miconazole from Janssen Pharmaceutica (Beerse, Belgium); and terbinafine from Sandoz Pharma (Basle, Switzerland). Amphotericin B was diluted in sterile, distilled water and the other powders in equal volumes of dimethylsulphoxide (DMSO; Janssen Chimica, Geel, Belgium) and sterile, distilled water. The ranges of dilutions were 0.03–64 $\mu g\ ml^{-1}$ for amphotericin B, 0.125–128 $\mu g\ ml^{-1}$ for the azoles and 0.03–128 $\mu g\ ml^{-1}$ for terbinafine.

The *Fusarium* strains were isolates from clinically apparent infections. They were identified by macroscopic and microscopic examination as follows: nine *F. solani*, four *F. moniliforme*, 10 *F. oxysporum*.

The inocula were made by rinsing a 12-day-old culture on diluted (1:10) Sabouraud glucose agar [4] with 3 ml of sterile, distilled water. These suspensions were inoculated on casitone agar by a swab in the case of the agar diffusion method and by a multipoint inoculator (Melrose Machine Products, Pennsylvania, USA.) in duplicate for the agar dilution method.

For the agar diffusion method the inhibition zone (in mm) was measured and for the agar dilution method the minimum inhibitory concentration (MIC) (in $\mu g\ ml^{-1}$) was considered the lowest concentration of the drug that prevented visible growth.

The plates for the agar diffusion method were incubated at 28 °C for up to 72 h and read every 24 h. The inhibition zones of the Neo-sensitabs loaded with azoles and 5-fluorocytosine could not be read after 24 h of incubation. After 48 h of incubation, there was no inhibition zone (9 mm = the diameter of the disc) or an inhibition zone containing a weak confluent growth. After 72 h of incubation, the confluent growth inside the inhibition zone did not differ from the confluent growth of the rest of the plate (no zone = 9 mm). In contrast, the Neo-sensitabs loaded with amphotericin B gave a clear inhibition zone without visible growth inside after 24 h and 48 h of incubation, and with terbinafine after 48 and 72 h of incubation.

The plates for the agar dilution method were read after 48 h and 72 h of incubation.

Results

The results of the agar diffusion and the agar dilution method for the 23 *Fusarium* strains are reported in Table 1. None of the strains showed an inhibition zone (9 mm) after 48 h incubation with the 5-fluorocytosine disc.

A zone on the fluconazole and itraconazole disc containing confluent growth had to be interpreted as resistant (MIC of fluconazole $> 128\ \mu g\ ml^{-1}$ and of itraconazole 64 to $> 128\ \mu g\ ml^{-1}$). The breakpoint of ketoconazole and miconazole is $\leq 8\ \mu g\ ml^{-1}$ [5]. In two *F. oxysporum* strains (48-h zones 19 and 20 mm with confluent growth inside) and one *F. moniliforme* strain (48-h zone 20 mm with confluent growth inside) the MIC of ketoconazole was $8\ \mu g\ ml^{-1}$. In four *F. oxysporum* strains, with 48-h zones of 12–18 mm containing confluent growth, ketoconazole had a MIC of 16–32 $\mu g\ ml^{-1}$. Seven *Fusarium* strains were inhibited by $\leq 8\ \mu g\ ml^{-1}$ miconazole (two *F. solani*, 48-h zone 9mm; one *F. moniliforme*, 48-h zone 9 mm; and four *F. oxysporum*, 2 with a 48-h zone of 17 mm with confluent growth inside and two with no zone). In one *F. oxysporum* strain, with a 48-h zone of 14 mm and confluent growth inside, miconazole had a MIC of 16 $\mu g\ ml^{-1}$.

The peak serum concentration of amphotericin B considered to be achievable is 1–2 $\mu g\ ml^{-1}$, and the cut-off point of susceptibility is $\leq 1.56\ \mu g\ ml^{-1}$ [5]. All but one *F. solani* strains were inhibited by $\leq 1\ \mu g\ ml^{-1}$ amphotericin B, 4/10 *F. oxysporum* strains were inhibited by $\leq 2\ \mu g\ ml^{-1}$ and none of the *F. moniliforme* strains tested was inhibited by concentrations $\leq 2\ \mu g\ ml^{-1}$. The zones of amphotericin B Neo-sensitab were difficult to interpret and varied among the three species tested: *F. moniliforme* had 24-h zones ≥ 13 mm and MICs $> 4\ \mu g\ ml^{-1}$; 24-h zones of ≥ 13 mm for *F. solani* (six strains) corresponded to MICs $\leq 1\ \mu g\ ml^{-1}$; four strains of *F. oxysporum* with MICs $\leq 2\ \mu g\ ml^{-1}$ had 24-h inhibition zones ≥ 16 mm. One of the other three strains of *F. solani* had a 24-h inhibition zone of 9 mm and a MIC of $1\ \mu g\ ml^{-1}$; the other two strains had 24-h zones of 10 mm and the MICs of amphotericin B were 1 and $4\ \mu g\ ml^{-1}$. The other six strains of *F. oxysporum* had 24-h zones between 12 and 15 mm and the MICs of amphotericin B were $4\ \mu g\ ml^{-1}$ (five strains) and $8\ \mu g\ ml^{-1}$ (one strain with a 24-h zone of 13 mm).

The 48-h inhibition zone of terbinafine Neo-sensitab of > 15 mm correlated with a MIC of

Table 1. Zones (mm) and corresponding MIC ranges (μg ml^{-1}) of antifungal agents for *Fusarium* spp.

Agent	n	*Fusarium* sp.	Zone (mm) after various incubation times			MIC range (μg ml^{-1})
			24 h*	48 h	72 h*	48 h
Fluconazole		*F. solani*		9	9	>128
	4	*F. moniliforme*		9	9	>128
	6	*F. oxysporum*		9	9	>128
	4	*F. oxysporum*		15†–23†	9	>128
Itraconazole	9	*F. solani*		9	9	>128
	4	*F. moniliforme*		9	9	>128
	8	*F. oxysporum*		9	9	64–>128
	2	*F. oxysporum*		12†–13†	9	>128
Ketoconazole	9	*F. solani*		9	9	32–64
	3	*F. moniliforme*		9	9	64
	1	*F. moniliforme*		20†	9	8
	4	*F. oxysporum*		9	9	16
	4	*F. oxysporum*		12†–18†	9	16–32
	2	*F. oxysporum*		19†–20†	9	8
Miconazole	7	*F. solani*		9	9	16–64
	2	*F. solani*		9	9	4–8
	3	*F. moniliforme*		9	9	32–64
	1	*F. moniliforme*		9	9	2
	5	*F. oxysporum*		9	9	16–32
	1	*F. oxysporum*		14†	9	16
	2	*F. oxysporum*		9	9	2–4
	2	*F. oxysporum*		17†	9	2–8
Amphotericin B	1	*F. solani*	10	9		4
	2	*F. solani*	9–10	10–11		1
	6	*F. solani*	13–17	9–17		0.250–1
	4	*F. moniliforme*	13–18	11–15		4–16
	6	*F. oxysporum*	12–15	9–14		4–8
	4	*F. oxysporum*	16–18	11–20		1–2
Terbinafine	5	*F. solani*		9–15	9–15	4–>128
	4	*F. solani*‡		17†–20	12(†)–17	1–2
	4	*F. moniliforme*		20–25	19–24	0.50–1
	10	*F. oxysporum*		20–28	18–28	0.250–2

* The inhibition zones of the Neo-sensitabs loaded with azoles and terbinafine could not be read after 24 h of incubation; those loaded with amphotericin B gave clear inhibition zones after 24 h of incubation.
† Zone with confluent growth inside; 9 mm = zone of the disc.
‡ One strain 17†/17† mm, two strains 17/15† mm, one strain 20–12 mm.

≤2 μg ml^{-1} for all strains tested. The highest plasma concentration of terbinafine after oral administration of 250 mg daily for 28 days is 1.39 μg ml^{-1} [6].

Discussion

The zones achieved with Neo-sensitabs loaded with fluconazole, itraconazole, ketoconazole and miconazole after 48 h of incubation gave major interpretation problems if a zone with confluent growth inside was measured. After 72 h of incubation no strain showed any zone (9 mm). Disc diffusion with Neo-sensitabs loaded with fluconazole, itraconazole, ketoconazole and miconazole gave false susceptible as well as false resistant results in comparison with the agar dilution method. A zone on fluconazole or itraconazole discs containing confluent growth must be interpreted as resistant. In conclusion, our results suggest that a ketoconazole zone ≥19 mm or a miconazole zone ≥17 mm with confluent growth inside after 48 h of incubation could indicate a sensitive strain.

The diameter of the zones measured after 24 h of incubation with amphotericin B Neo-sensitab varied greatly between the three species tested: in *F. solani* with zones of >12 mm MICs were ≤1 μg ml^{-1}, in *F. oxysporum* with zones of >15 mm MICs were ≤2 μg ml^{-1} and in *F. moniliforme* with zones of >12 mm MICs were

≥ 4 μg ml^{-1}. Inhibition zones after 48 h of incubation with terbinafine Neo-sensitabs of >15 mm correlated with MICs of ≤ 2 μg ml^{-1}.

All *Fusarium* strains were highly resistant to fluconazole and itraconazole. Miconazole showed some activity (2/9 *F. solani*, 1/4 *F. moniliforme* and 4/10 *F. oxysporum*), whereas ketoconazole was poorly active (1/4 *F. moniliforme* and 2/10 *F. oxysporum*). These results for the azoles are similar to the results of the many case reports in which a single or a few strains of *Fusarium* were tested *in vitro* [2] and to those of studies investigating a greater number of strains [7, 8]. Intravenous amphotericin B has been the most commonly used antifungal agent in disseminated *Fusarium* infections, although the susceptibility of the isolates to amphotericin B varies [2, 7, 8]. In our study, amphotericin B was more active against the *F. solani* strains (8/9) than against the *F. oxysporum* strains (4/10) and had no activity against the *F. moniliforme* strains tested. Sekhon *et al.* [8] found only 1 of 16 *Fusarium* strains to be sensitive to amphotericin B. Reuben *et al.* [7] found all 44 *Fusarium* spp. tested to be susceptible to amphotericin B.

Terbinafine showed very good activity *in vitro* against *F. moniliforme* strains (4/4) and *F. oxysporum* strains (10/10), but was less active active against *F. solani* strains (4/9). At present, there are no reports of cases of systemic *Fusarium* infections treated with terbinafine.

MICs, especially these of miconazole and amphotericin B, read after 72 h of incubation, had values that were one or two dilutions higher than those read after 48 h of incubation.

In its manual for antifungal sensitivity testing using Neo-sensitabs, Rosco Diagnostica presents only zone interpretative standards when testing yeasts. It does not provide any information regarding sensitivity testing of moulds. In this study we obtained different results using the disc diffusion and the agar dilution methods for sensitivity testing of 23 clinical isolates of *Fusarium* spp. In contrast, Casals [3] found a good correlation between agar diffusion with Neo-sensitab tablets and an agar dilution method using 80 strains of fungi, including yeasts and *Aspergillus fumigatus* (for example, a zone of amphotericin B of ≥ 15 mm correlated with a MIC of ≤ 1 μg ml^{-1}). The inhibitory zones for *Fusarium* spp. achieved when using the rather practical sensitivity test with Neo-sensitabs need very careful interpretation.

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