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In-vitro susceptibility of *Aspergillus* spp. isolates to amphotericin B and itraconazole

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The MICs of amphotericin B and itraconazole for 230 isolates of *Aspergillus* spp., comprising 156 *Aspergillus fumigatus*, 20 *Aspergillus terreus*, 22 *Aspergillus flavus*, 17 *Aspergillus nidulans* and 15 *Aspergillus niger*, were determined by a broth microdilution method with RPMI 1640 medium. No isolate was detected with an MIC of amphotericin B >2 mg/L. Itraconazole MICs >16 mg/L were detected for four *Aspergillus fumigatus* and one *Aspergillus nidulans* isolates.

Introduction

Severe fungal infections have increased markedly in recent years, and *Aspergillus* spp. are the most frequent filamentous fungi causing invasive infections in immunocompromised patients. Amphotericin B and itraconazole are currently the only two drugs available for treatment of invasive aspergillosis.

Although reference methods for susceptibility testing of yeasts have been developed, susceptibility testing of filamentous fungi to antifungal drugs is not well standardized, and testing conditions are still being evaluated.[1] *In vitro*, resistance of *Aspergillus* spp. to itraconazole has been described recently,[2,3] but the frequency of this resistance is largely unknown. Moreover, correlation between these in-vitro results and in-vivo outcome in animal models with some *Aspergillus* spp. strains showed that resistance detected *in vitro* could have clinical relevance.[3,4]

The current study was undertaken to determine the distribution of amphotericin B and itraconazole MICs by testing a large number of clinical isolates of *Aspergillus* spp. belonging to the five major species recovered from human infections.

Materials and methods

Organisms

Two-hundred-and-thirty isolates from 130 patients, comprising 156 *Aspergillus fumigatus*, 20 *Aspergillus terreus*, 22 *Aspergillus flavus*, 17 *Aspergillus nidulans* and 15 *Aspergillus niger*, were tested. The isolates were collected from 130 patients between January 1996 and August 1998, and were stored as conidial suspensions at –80°C in 10% glycerol until testing was initiated. An isolate of *Candida krusei* (ATCC 6258) was included as a control in each set of MIC determinations.

Susceptibility testing

Amphotericin B (Sigma, St Louis, MO, USA) and itraconazole (Janssen Pharmaceutica, Beerse, Belgium) were dissolved in dimethylsulphoxide (Sigma) to a concentration of 1600 mg/L and stored at –80°C as stock solutions.

Susceptibility testing was performed using a NCCLS-based broth microdilution technique.[1] RPMI 1640 (Gibco-BRL, Uxbridge, UK) with L-glutamine and without sodium bicarbonate, and buffered at pH 7.0 with 0.165 M morpholinepropanesulphonic acid (MOPS), was used as test medium. The 2 × drug dilutions (32 to 0.06 mg/L) were prepared with medium used as the diluent, and dispensed into rows two to 11 of 96 U-shaped well microplates in 100 μL volumes. Row one contained 200 μL of uninoculated, drug-free medium and the wells of row 12 were used as growth controls.

The isolates were grown on malt extract agar (MEA) slants (Sanofi Diagnostics Pasteur, Marnes La Coquette, France) for 5 days at 35°C. The isolate of *C. krusei* (ATCC 6258) was grown on the same agar for 24 h at 35°C. The surface of the agar slants were washed over with 1 mL of

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sterile 0.9% saline containing 0.05% Tween 80, and the conidial suspensions were counted manually with a haemocytometer. The conidia were diluted in RPMI to produce a working suspension of 2×10^4 conidia/mL. Each well of rows two to 12 was inoculated with 100 μL of the $2 \times$ conidial suspension. Hence, the final drug concentrations were 0.03–16 mg/L for both drugs, and the final inoculum concentration was 10^4 conidia/mL. The MICs for all isolates were determined in duplicate. Microplates were incubated at 35°C for 48 h, and the growth in each well was compared with that of the growth control with the aid of a reading mirror. Each well was then given a numerical score from four (no reduction in growth) to zero (absence of growth).[1]

MIC endpoints were defined as the lowest drug concentration to have a score of one for itraconazole and zero for amphotericin B. The difference in the distributions of amphotericin B and itraconazole MICs was determined with a one-way analysis of variance.

Results and discussion

The MIC ranges, MIC_{50}s and MIC_{90}s of both drugs against the five *Aspergillus* spp. are presented in the Table.

The MICs of amphotericin B ranged from 0.12 to 2 mg/L. *A. fumigatus* and *A. niger* showed the lowest MICs of amphotericin B, and there was no difference between the MICs for these two species ($P > 0.05$). *A. flavus*, *A. nidulans* and *A. terreus* were significantly less susceptible to amphotericin B than *A. fumigatus* ($P < 0.001$, $P < 0.01$ and $P < 0.001$, respectively) and than *A. niger* ($P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively). Previously, similar results have been found in studies using microdilution techniques with RPMI medium.[5] No isolates had an MIC >2 mg/L for amphotericin B in this study. This is not surprising as it is well recognized that use of the NCCLS M27 method[6] to test amphotericin B against *Candida* spp. results in a very narrow range of MIC.[5] Recently, in-vivo resistance to amphotericin B has been demonstrated in an animal model of invasive aspergillosis for one isolate of *A. fumigatus*, but standard in-vitro susceptibility tests were unable to confirm the observed in-vivo resistance.[7] Therefore, amphotericin B resistance among *Aspergillus* spp. remains largely unknown, and the development of a reliable in-vitro susceptibility testing technique needs to be addressed.

The MICs of itraconazole ranged from 0.12 to >16 mg/L. There were no statistically significant differences between paired species for itraconazole. MICs >16 mg/L were detected for four *A. fumigatus* isolates and one *A. nidulans* isolate. These MICs were reproducible and the srains were considered to be resistant.

One *A. fumigatus* isolate resistant to itraconazole was cultured from the sputum of a patient who was treated with amphotericin B and 5-fluorocytosine for an *Aspergillus* spp. brain abcess. This patient had not received itraconazole before the strain was isolated. A second *A. fumigatus* isolate resistant to itraconazole was cultured from a patient with aspergilloma. This patient received itraconazole for several months before the strain was isolated. The two other resistant *A. fumigatus* isolates were cultured from broncho-alveolar lavages from a patient treated with itraconazole for a chronic necrotizing pulmonary aspergillosis. From this patient, *A. fumigatus* isolates with an itraconazole MIC of 0.5 mg/L were cultured initially, and after 4 months of treatment with itraconazole, two isolates were found to be resistant. This case suggests either the possibility of development of resistance, or secondary contamination by an itraconazole-resistant strain. Genomic analysis of these isolates will be necessary to clarify this.

Several studies have shown that in-vitro activity of itraconazole against *A. fumigatus* is high, and in some of these studies no itraconazole-resistant isolates have been identified.[8,9] Nevertheless, in a large series using an NCCLS procedure, it was reported that three patients were infected with *A. fumigatus* strains that acquired in-vitro resistance to itraconazole (MIC > 32 mg/L) during prolonged therapy.[2] Moreover, three itraconazole-resistant strains of *A. fumigatus* from two patients have been recently detected and the resistance has been confirmed in an animal model.[3] Our data confirm previous studies demonstrating in-vitro resistance of *A. fumigatus* to itraconazole, although the frequency of this resistance appears to be low.

Table. MICs of amphotericin B and itraconazole for the different *Aspergillus* spp.

Organism (no. of isolates)	Amphotericin B MIC (mg/L)			Itraconazole MIC (mg/L)		
	range	MIC_{50}	MIC_{90}	range	MIC_{50}	MIC_{90}
A. fumigatus (156)	0.12–2	0.5	1	0.12–>16	0.5	2
A. flavus (22)	0.5–2	1	2	0.12–1	0.25	0.5
A. nidulans (17)	0.5–2	1	2	0.12–>16	0.25	4
A. niger (15)	0.12–0.5	0.5	0.5	0.12–1	0.5	1
A. terreus (20)	0.25–2	2	2	0.25–1	0.5	1
Total (230)	0.12–2	1	2	0.12–>16	0.5	2

The *A. nidulans* isolate was cultured from a patient who did not receive any antifungal treatment, suggesting that primary itraconazole resistance can be detected in non-*fumigatus Aspergillus* spp.

Further studies with a larger number of isolates are required to establish the frequency of itraconazole resistance for the different *Aspergillus* spp. The in-vitro data obtained in this and other studies[2,3] suggest that *Aspergillus* spp. can be resistant to itraconazole. Since there is little information regarding the clinical relevance of in-vitro data for moulds, these results should first be validated *in vivo*.

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