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In Vitro Activities of Amphotericin B, Terbinafine, and Azole Drugs against Clinical and Environmental Isolates of *Aspergillus terreus Sensu Stricto*

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The antifungal susceptibilities of 40 clinical and environmental isolates of *A. terreus sensu stricto* to amphotericin B, terbinafine, itraconazole, and voriconazole were determined in accordance with CLSI document M38-A2. All isolates had itraconazole and voriconazole MICs lower than epidemiologic cutoff values, and 5% of the isolates had amphotericin B MICs higher than epidemiologic cutoff values. Terbinafine showed the lowest MICs. No significant differences were found when MICs of clinical and environmental isolates were compared.

In recent decades, fungal infections due to *Aspergillus* species have become a major cause of morbidity and mortality among immunocompromised patients (1–3). *Aspergillus fumigatus* is the most frequently isolated species, although there has been an increase in the incidence of other species, including *Aspergillus flavus*, *A. niger*, and *A. terreus* (1, 4, 5).

A. terreus is considered an emerging opportunistic fungus which can produce superficial to serious invasive infections (4–8). Invasive infections are often treated empirically with amphotericin B, a widely used broad-spectrum drug. However, most *A. terreus* isolates are resistant *in vivo* and *in vitro* to this drug (9–13).

Voriconazole has proved to be most effective, *in vivo* and *in vitro*, against this species (14–16), although some publications (9, 17–19) have already reported clinical isolates of *A. terreus* with higher MICs than the established epidemiologic cutoff values (ECVs) for itraconazole and voriconazole (20).

The aim of this study was to determine the antifungal susceptibility profile of clinical and environmental isolates of *A. terreus* for amphotericin, terbinafine, and triazole derivatives and monitor the possible emergence of strains with reduced antifungal triazole activity.

A total of 40 isolates of *A. terreus* complex—19 clinical and 21 environmental—were studied. Environmental isolates were obtained from indoor and outdoor hospital environments and from soils and trees in Resistencia (27°27′05″S, 58°59′12″W) and Corrientes (27°30′00″S, 58°48′00″W) (cities located in northeastern Argentina). Clinical isolates were obtained from skin and soft tissues samples, bronchoalveolar lavage samples, fingernails, and toenails.

All of isolates were identified as *A. terreus* complex according to general taxonomical keys (21–24).

For molecular identification, DNA extraction was performed according to the method described by Bosco Borgeat et al. (25) The partial sequence of the calmodulin (CalM) gene was amplified under conditions described by Peterson (26), using primers CF1 F (5′GCCGACTCTTTGACYGARGAR) and CF4 R (5′TTTYTGCA TCATRAGYTGGAC). PCR products were purified using PureLink quick PCR purification kit (Invitrogen, Germany) following the supplier's protocol. PCR products were sent for sequencing to the Department of Ecology, Genetics and Evolution Sequencing and Genotyping Service, University of Buenos Aires, Buenos Aires, Argentina, and to the Division of Hygiene and Medical Microbiology Medical University of Innsbruck, Innsbruck, Austria. Bidirectional sequencing was performed for all isolates. Sequencing errors were detected and corrected with BioEdit sequence alignment editor software, version 7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). All of the isolates were identified as *A. terreus sensu stricto*.

MICs were determined by broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) document M38-A2 (27).

Antifungal activities were determined for voriconazole (Pfizer, USA), itraconazole (Sigma-Aldrich, Argentina), terbinafine (Sigma-Aldrich, Argentina), and amphotericin B (Sigma-Aldrich, Argentina). Solutions were prepared in dimethyl sulfoxide (Sigma-Aldrich, Argentina) and stored at −70°C until they were used. The final concentrations of all drugs were 0.03 to 16 μg/ml.

The quality control strains *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, and *A. flavus* ATCC 204304 were included in each testing assay (27).

MIC endpoints for amphotericin B, azoles, and terbinafine were considered the lowest concentrations that produced a complete inhibition of visible growth at 48 h.

The significance of the differences in MICs between clinical

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TABLE 1 MICs of amphotericin B, terbinafine, and azole drugs obtained by broth microdilution for 40 *Aspergillus terreus sensu stricto* isolates

Isolate type (no. tested)	Drug[a]	MIC (μg/ml)					% ≤ECV[b]
		Range	GM[c]	Mode	MIC_{50}	MIC_{90}	
Clinical (19)	AMB	1–8	2.17	2	2	4	94.74
	VRC	0.125–0.5	0.30	0.25	0.25	0.5	100
	ITC	≤0.03–0.5	0.26	0.5	0.125	0.5	100
	TER	≤0.03–0.25	0.14	0.25	0.125	0.25	ND[b]
Environmental (21)	AMB	1–8	2.24	2	2	4	95.24
	VRC	0.125–0.5	0.41	0.5	0.5	0.5	100
	ITC	≤0.03–0.5	0.21	0.25	0.25	0.5	100
	TER	≤0.03–0.25	0.09	0.125	0.06	0.125	ND[b]

[a] AMB, amphotericin B; VRC, voriconazole; ITC, itraconazole; TER, terbinafine.
[b] Percentage of MICs less than or equal to than the ECV (ECV = 1 μg/ml for itraconazole and voriconazole and 4 μg/ml for amphotericin B). ND, not determined (no ECVs were available for TER).
[c] GM, geometric mean.

and environmental isolates was determined by the Student *t* test (unpaired, unequal variance). A *P* value of <0.05 was considered significant.

The MIC ranges, geometric means, modes, MIC_{50}s, and MIC_{90}s obtained are summarized in Table 1.

A. terreus is a cosmopolitan fungus frequently isolated from indoor and outdoor environments in northeast Argentina (28). In addition, it is one of the more frequently opportunistic agents of onychomycosis isolated in these regions (22).

Clinical breakpoints have not been established for mold testing. However, epidemiologic cutoff values (ECVs) of and amphotericin B, itraconazole, posaconazole, and voriconazole are available for five *Aspergillus* spp. (among them *A. terreus*). The ECV of amphotericin B for *A. terreus* was defined as 4 μg/ml, encompassing 97.5% of the modeled wild-type population (29), and the ECVs of itraconazole and voriconazole for *A. terreus* were defined as 1 μg/ml (20).

The use of voriconazole for the treatment of invasive aspergillosis caused by *A. terreus* improved clinical response of patients (15, 16, 29, 39). These *in vivo* results correlate with our *in vitro* data; all voriconazole MICs were lower than the ECV (20) for both clinical and environmental isolates. The same situation was observed for itraconazole; some strains even showed MICs lower than that for voriconazole. Similar results were reported by other authors, showing a high *in vitro* activity of these antifungals (30–33). In contrast, some reports from European countries and the United States describe strains of *A. terreus* with MICs higher than the ECV for voriconazole and itraconazole (9, 17, 18, 20, 31).

Reports on susceptibility testing of terbinafine have increased since this antifungal has shown a high activity *in vitro* against a broad spectrum of pathogenic fungi (34). This drug showed potent *in vitro* activity against all isolates of *A. terreus* tested, with MICs lower than triazole derivates. These data are consistent with values published by Moore and Walls, who reported a MIC_{90} of 0.25 μg/ml with a range of 0.125 to 1 μg/ml (11). Garcia-Effron et al. reported higher values (MIC_{90}, 1 μg/ml; range, 0.03 to 4 μg/ml) (34), although these differences may be due to the different methods used.

Most investigations show that *A. terreus* has intrinsic resistance to amphotericin B, with elevated MICs (4, 9, 14, 32, 34, 35). In our study, 95% (38/40) of all isolates exhibited amphotericin B MICs of ≤4 μg/ml. Only two isolates (one clinical and one environmental) showed amphotericin B MICs of 8 μg/ml, above the proposed ECV (29). On the other hand, some studies have reported strains with low MICs (<1 μg/ml) for this drug (18, 30, 31, 36). Only 4/19 clinical isolates and 8/21 environmental isolates showed amphotericin B MICs of 1 μg/ml in our study. These findings suggest that there may be *A. terreus sensu stricto* strains that are susceptible to amphotericin B, but more research is needed to know if these isolates represent variants with susceptibility to amphotericin B.

No statistically significant differences between the susceptibility data obtained for clinical and environmental isolates were observed, as reported by other authors (30, 37), although Araujo et al. (38) found environmental isolates with significantly higher MICs than clinical isolates for amphotericin B.

Antifungal susceptibility testing is essential in patient management and surveillance of resistance. Little is known about the susceptibility profile of *A. terreus* worldwide. The present study is a contribution to the knowledge of the susceptibility of this opportunistic fungus and shows that *A. terreus sensu stricto* isolates obtained in this region have low MICs for itraconazole, voriconazole, and terbinafine and exhibit high amphotericin B MICs.

Nucleotide sequence accession numbers. Sequences of the CalM genes of the 40 *A. terreus* isolates have been submitted to the European Nucleotide Archive (ENA) and assigned the accession numbers LN734824 to LN734863 (http://www.ebi.ac.uk/ena/data/view/LN734824-LN734863).

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We have no conflict of interest to declare.

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