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MICs and minimum fungicidal concentrations of amphotericin B, itraconazole, posaconazole and terbinafine in *Sporothrix schenckii*

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The *in vitro* susceptibility of 62 isolates of *Sporothrix schenckii* in its mycelial form, from Latin-American countries (Peru, Venezuela, Brazil and Uruguay) and Spain, to amphotericin B (AB), itraconazole (IZ), posaconazole (PZ) and terbinafine (TB) was determined by measuring the MICs and minimum fungicidal concentrations (MFCs) using a standardized Clinical and Laboratory Standards Institute method. In general, TB was the most active drug, with the lowest geometric mean (GM) MIC and MFC values amongst isolates from the five countries tested. IZ and PZ showed almost the same activity against all strains tested, except for isolates from Uruguay where IZ gave the highest GM MIC (10.68 mg l^{-1}). AB showed the widest MIC range (0.03–16.0 mg l^{-1}); however, this drug was less active against 79 % of isolates (MICs above 1 mg l^{-1}). MFCs were 5 to 20 times higher than the MICs, but the lowest GM MFC and range values were found for TB. IZ and PZ gave the highest GM MFC. MFC may be a better predictor of therapeutic response than MIC, especially in immunosuppressed patients, making the use of IZ and PZ an inappropriate treatment. There were some differences in susceptibility according to the geographical source of the isolates, with the MIC being lower for TB in Venezuelan strains (P=0.066) and the MFC higher for PZ in Peruvian strains (P=0.02). Thus, geographical origin may be important for appropriate treatment, and may relate to the identification of species of the *S. schenckii* complex.

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INTRODUCTION

Sporothrix schenckii, now considered as a complex (Marimon *et al.*, 2007), is a dimorphic pathogenic fungus that is the aetiological agent of human and animal sporotrichosis. This species is widely distributed in nature and can be found in soil associated with organic plant matter (Ramos-e-Silva *et al.*, 2007). The most frequent clinical manifestation is the subacute or chronic cutaneous–lymphatic form, followed by fixed cutaneous infection (Bonifaz *et al.*, 2007). More severe systemic presentations affecting joints, lungs and the central nervous system, as well disseminated infections, have also been described, especially in immunosuppressed patients (Vilela *et al.*, 2007).

Traditional treatment of localized cutaneous–lymphatic sporotrichosis is a saturated solution of potassium iodine given orally for 2–3 months (Kauffman, 1995). The availability of some active systemic antifungals such as ketoconazole, itraconazole (IZ) and terbinafine (TB) allows effective treatment (Chapman *et al.*, 2004) that avoids the secondary effects of iodine. Amphotericin B (AB) has been used for visceral and disseminated forms with varying results (Baker *et al.*, 1989; Kosinski *et al.*, 1992; Kohler *et al.*, 2007). Among the newest antifungals, posaconazole (PZ) seems to be active against *S. schenckii* but limited information on a low number of isolates is available (Scheinfeld, 2007; Marimon *et al.*, 2008).

Abbreviations: AB, amphotericin B; GM, geometric mean; IZ, itraconazole; MFC, minimum fungicidal concentration; PZ, posaconazole; TB, terbinafine.

The aim of this study was to determine the MICs of AB, IZ, TB and PZ for clinical strains of *S. schenckii* using the standardized broth microdilution method M38-A recommended by the Clinical and Laboratory Standards Institute (formerly the National Committee on Clinical Laboratory Standards) (NCCLS, 2002). Minimum fungicidal concentrations (MFCs) were also measured to determine the killing power of these antifungals against the mycelial phase of *S. schenckii*.

METHODS

Strains. *In vitro* tests were performed on 62 clinical isolates of *S. schenckii*, cultivated from patients with cutaneous or cutaneous–lymphatic sporotrichosis from different countries. Strains were isolated in Peru (n=36), Venezuela (n=15), Brazil (n=6), Uruguay (n=3) and Spain (n=2). The isolates were conserved in a skimmed milk suspension at –20 °C until use.

Antifungal drugs. According to the manufacturer's instructions, AB (Sigma-Aldrich), PZ (Schering-Plough), IZ (Janssen Research Foundation) and TB (Novartis) were dissolved in 100 % DMSO, and diluted in RPMI 1640 buffered to pH 7.0 with 0.165 M 3-(*N*-morpholino)propanesulfonic acid, and supplemented with glucose (18 g l^{-1}) (Alvarado-Ramírez & Torres-Rodríguez, 2007). Drug concentrations were 0.03–16.0 mg l^{-1} for all drugs. Serial dilutions of each drug were placed in microdilution plates and stored at –30 °C until use.

Broth microdilution test. Isolates were tested using the microbroth dilution method M38-A (NCCLS, 2002) with slight modification (Alvarado-Ramírez & Torres-Rodríguez, 2007). Inoculum was obtained by growing the isolates for 5 days at 28±2 °C in potato dextrose agar. *Aspergillus flavus* ATCC 204304 was used for quality control. To prepare the inoculum, conidia were removed from the colony surface with 3–4 ml sterile saline solution by gently scraping the surface. The stock suspension was diluted in saline solution and adjusted spectrophotometrically to an OD_{530} of 0.05–0.1 (80–82 % transmittance), corresponding to 1×10^5 c.f.u. ml^{-1}. The viability of each isolate was confirmed by inoculation to Sabouraud dextrose agar plates and counting the number of c.f.u. ml^{-1}. The MIC end-point criterion was the lowest drug concentration at which there was no visible growth after 72 h incubation at 28 °C. To obtain the MFC, 10 µl of each serial dilution was taken from each well and spread on Sabouraud dextrose agar. Plates were incubated at 28 °C for 72 h. The MFC was defined as the lowest drug concentration that yielded three or fewer colonies (i.e. 99 % of the inoculum was killed) (Espinel-Ingroff *et al.*, 2002).

Statistical analysis. The Wilcoxon rank-sum test was used for statistical analysis, with statistical significance being set at $P<0.05$. Results were analysed using the software SPSS v13.0 for Windows (SPSS).

RESULTS AND DISCUSSION

The geometric means (GMs), range of maximum and minimum values, and 50 and 90 % MIC/MFC values were determined. Table 1 shows the MICs and MFCs for the four antifungals for the 62 *S. schenkii* isolates from the five country group values. MIC values for the reference *A. flavus* strains were within the limits described for AB and IZ (4 and 0.5 mg l^{-1}, respectively).

In this study, 64 % of *S. schenkii* were susceptible to IZ at an MIC of 0.5 mg l^{-1}. The strains from Uruguay showed the highest GM (10.68 mg l^{-1}); however, we tested only three isolates and two were resistant. MFCs were higher, with GMs (mg l^{-1}) of 14.44 (Peru), 11.73 (Venezuela), 14.67 (Brazil), 11.33 (Uruguay) and 16 (Spain). Other authors (McGinnis *et al.*, 2001) have demonstrated good activity of IZ in terms of MIC and MFC values against 100 isolates of *S. schenckii*. This is in agreement with our MIC results; however, our results showed that IZ had a high MFC for most isolates of *S. schenckii*.

For PZ, MIC values were similar to IZ; the strains from Uruguay showed the highest MFCs, and the strains from Brazil showed a lower GM of 10 mg l^{-1}. The treatment therapies used in Peru are potassium iodide, ketoconazole, IZ and TB (ongoing clinical trial) (Pappas *et al.*, 2000). There are no data on the susceptibility of Peruvian *S. schenckii* isolates to PZ. TB gave the lowest MIC and MFC values.

As found elsewhere (Alvarado-Ramírez & Torres-Rodríguez, 2007; Ellis, 2002), AB showed a wide susceptibility range. The isolates from Peru (GM 1.76 mg l^{-1}), Uruguay (GM 1.67 mg l^{-1}), Brazil (GM 2.17 mg l^{-1}) and Spain (GM 2 mg l^{-1}) presented the highest MICs. MFCs were higher than MICs (Table 1). If AB is the treatment of choice, the MIC of the specific isolate should be determined, as failure of treatment could be associated with the lower susceptibility of *S. schenckii* to this antifungal (McGinnis *et al.*, 2001).

Statistical differences were found according to the geographical source of the isolates for the MIC and MFC, being lower for TB in Venezuelan strains (P=0.066) and higher for PZ in Peruvian strains (P=0.02), respectively.

Neyra *et al.* (2005) showed no correlation between genotype, geographical origin and clinical form in Peru. Mesa-Arango *et al.* (2002) also found no association between the virulence of *S. schenckii* and its geographical precedence. Marimon *et al.* (2007) analysed 127 isolates from different countries, including Brazil, Japan, Peru, Venezuela and the USA, and many proved to be genetically different from each other. Because of this, they proposed three new *Sporothrix* species, two of which have been associated with human infections (*Sporothrix brasiliensis* and *Sporothrix globosa*). The isolates of clinical origin were included in three clades: clade I included only Brazilian isolates and was classified as *S. brasiliensis*. Clade II included practically all the USA isolates (*S. schenckii*) and clade III was composed of isolates from China, India, Italy, Japan, Spain and the USA, and were named *S. globosa*. In that study, phenotypic characteristics were not found to distinguish isolates in clade II, despite this group being genetically heterogeneous. This suggests that our isolates may represent not only *S. schenckii*, but also probably another species such as *S. brasiliensis*, which has the same USA origin as the isolates of Marimon *et al.* (2007). It will be important to classify these isolates, as they show

Table 1. MICs, MFCs, range and susceptibilities (%) at various MICs of four antifungal drugs in strains of *S. schenckii,* in a mycelial form, from five countries

Group (*n*) and antifungal agent	MIC (mg l^{-1})				MFC (mg l^{-1})				Susceptibility (%) at MIC (mg l^{-1}) of:						
	GM	Range	50 %	90 %	GM	Range	50 %	90 %	⩽0.25	0.5	1	2	4	8	⩾16
Group 1 – Peru (36)															
AB	1.76	0.12–16	2	2	6.03	0.5–16	4	16	5.56	22.22	19.44	50	–	–	2.78
TB	0.37	0.03–1	0.5	0.5	1.97	0.25–8	1	4	47.22	50	2.78	–	–	–	–
IZ	0.54	0.25–1	0.5	1	14.44	4.0–16	16	16	13.89	72.22	13.89	–	–	–	–
PZ	0.67	0.25–1	0.5	1	14.83	2.0–16	16	16	8.33	55.56	36.11	–	–	–	–
Group 2 – Venezuela (15)															
AB	1.54	0.03–2	2	2	7.57	0.5–16	4	16	6.67	13.33	13.33	66.67	–	–	–
TB	0.27	0.03–0.5	0.25	0.5	1	0.5–2.0	1	2	73.33	26.67	–	–	–	–	–
IZ	0.6	0.03–1	0.5	1	11.73	2.0–16	16	16	6.67	66.67	26.67	–	–	–	–
PZ	0.67	0.03–1	0.5	1	12.13	2.0–16	16	16	20	33.33	46.67	–	–	–	–
Group 3 – Brazil (6)															
AB	2.17	1.0–4	2	2	11	2.0–6	8	16	–	–	16.67	66.67	16.67	–	–
TB	0.42	0.25–0.5	0.5	0.5	1.29	0.25–4	1	4	33.33	66.67	–	–	–	–	–
IZ	0.67	0.5–1	0.5	1	14.67	8.0–16	16	16	–	66.67	33.33	–	–	–	–
PZ	0.58	0.25–1	0.5	1	10	2.0–16	8	16	33.33	33.33	33.33	–	–	–	–
Group 4 – Uruguay (3)															
AB	1.67	1.0–2.0	2	2	6.67	2.0–16	2	16	–	–	33.33	66.67	–	–	–
TB	0.19	0.06–0.25	0.25	0.25	1.04	0.12–2	1	2	100	–	–	–	–	–	–
IZ	10.68	0.03–16	16	16	11.33	2.0–16	16	16	33.33	–	–	–	–	–	66.67
PZ	1.35	0.06–0.25	0.25	0.25	11	1.0–16	16	16	100	–	–	–	–	–	–
Group 5 – Spain (2)															
AB	2	2	2	2	6	4.0–8.0	4	8	–	–	–	100	–	–	–
TB	0.5	0.5	0.5	0.5	1.5	1.0–2.0	1	2	–	100	–	–	–	–	–
IZ	1.5	1.0–2.0	1	2	16	16	16	16	–	–	50	50	–	–	–
PZ	1.5	1.0–2.0	1	2	16	16	16	16	–	–	50	50	–	–	–
Total (62)															
AB	1.40	0.03–16.0	2.0	2.0	4.0	0.5–16.0	4.0	16.0	4.84	16.13	17.75	58.06	1.61	–	1.61
TB	0.29	0.03–1.0	0.25	0.5	1.0	0.12–8.0	1.0	4.0	53.23	45.16	1.61	–	–	–	–
IZ	0.51	0.03–2	0.5	1.0	10.7	2.0–16.0	16.0	16.0	11.29	64.52	19.35	1.61	3.23	–	–
PZ	0.64	0.03–2	0.5	1.0	9.6	1.0–16.0	16.0	16.0	12.9	45.16	37.1	4.84	–	–	–

different responses to antifungal agents that could be critical for appropriate patient management.

Recently, the susceptibilities of 92 isolates belonging to 5 species of *Sporothrix* were determined against 12 antifungal agents. TB, ketoconazole and PZ were shown to be the most active drugs. The authors suggested that PZ is a better therapeutic agent in the treatment of systemic infections than AB and IZ, which gave high MICs (Marimon *et al.*, 2008). These isolates in general gave MICs higher than our strains for IZ, AB and PZ, indicating that antifungal susceptibilities are strain-dependent. In contrast, for TB, the MICs were very similar to those seen in this study.

Our results suggest that some differences may exist in susceptibility to some antifungals (TB and PZ) according to the geographical origin of the strains. Further studies are needed, with larger numbers of isolates from other countries and molecular typing of these isolates to investigate whether they are genetically different or not and whether different species in the *S. schenckii* complex are important, to confirm these findings.

The results of this study support the therapeutic use of TB as a first option for the treatment of sporotrichosis (Chapman *et al.*, 2004) and suggest consideration of the MFC values for IZ before starting treatment. PZ could be another alternative treatment, but further clinical studies are necessary before proposing this drug as a therapeutic option.

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